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Bioactives from bitter melon enhance insulin signaling and modulate acyl carnitine content in skeletal muscle in high-fat diet-fed mice☆,☆☆

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Abstract

Bioactive components from bitter melon (BM) have been reported to improve glucose metabolism *in vivo*, but definitive studies on efficacy and mechanism of action are lacking. We sought to investigate the effects of BM bioactives on body weight, muscle lipid content and insulin signaling in mice fed a high-fat diet and on insulin signaling in L6 myotubes. Male C57BL/6J mice were randomly divided into low-fat diet control (LFD), high-fat diet (HFD) and HFD plus BM (BM) groups. Body weight, body composition, plasma glucose, leptin, insulin and muscle lipid profile were determined over 12 weeks. Insulin signaling was determined in the mouse muscle taken at end of study and in L6 myotubes exposed to the extract. Body weight, plasma glucose, insulin, leptin levels and HOMA-IR values were significantly lower in the BM-fed HFD group when compared to the HFD group. BM supplementation significantly increased IRS-2, IR β, PI 3K and GLUT4 protein abundance in skeletal muscle, as well as phosphorylation of IRS-1, Akt1 and Akt2 when compared with HFD ($P<.05$ and $P<.01$). BM also significantly reduced muscle lipid content in the HFD mice. BM extract greatly increased glucose uptake and enhanced insulin signaling in L6 myotubes. This study shows that BM bioactives reduced body weight, improved glucose metabolism and enhanced skeletal muscle insulin signaling. A contributing mechanism to the enhanced insulin signaling may be associated with the reduction in skeletal muscle lipid content. Nutritional supplementation with this extract, if validated for human studies, may offer an adjunctive therapy for diabetes.

Keywords: Bitter melon extract; Acyl carnitine; IRS-1; PI 3Kinase; High-fat diet

1. Introduction

Type 2 diabetes, a leading cause of death in the United States, has reached epidemic proportions in the US and worldwide (>18 million and 160 million individuals, respectively), and is projected to increase dramatically [1]. Furthermore, the prevalence of insulin resistance, a major pathophysiologic parameter contributing to development of type 2 diabetes and an independent risk factor for cardiovascular disease and the metabolic syndrome, is even more widespread [2,3]. Thus, the prevention and treatment of diabetes and obesity present major challenges to the health care system over the coming decades.

As an alternative therapy for diabetes, bitter melon (BM) (*Momordica charantia*) has been used historically in Asia, Africa and Latin America, because of its reported hypoglycemic activity [4]. BM has been proposed to contain bioactive components having anti-diabetic properties such as charantin, vicine and polypeptide-p, as well as other unspecific bioactive components including antioxidants. The metabolic and hypoglycemic effects of BM extracts have been demonstrated in cell culture, animal and human studies [5,6]. Recently, a study observed that BM extract was effective in ameliorating fructose diet-induced hyperglycemia, hyperleptinemia, hyperinsulinemia and hypertriglyceridemia as well as in decreasing the levels of free fatty acid in preclinical studies. The mechanism was proposed to be secondary to significantly increasing the expression of

Abbreviations: IPGTT, intraperitoneal glucose tolerance test; IRS-1, insulin receptor substrate 1; GLUT4, glucose transporter 4.

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☆☆ Z.Q. Wang and W.T. Cefalu designed the research; X.H Zhang and Y.M Yu conducted the research experiments; D. Ribnicky and A. Poulev analyzed BM extract by using HPLC/LC-MS; Z.Q. Wang performed the statistical analysis and wrote the manuscript, and Z.E. Floyd and W.T. Cefalu reviewed and edited the manuscript. Z.Q. Wang had primary responsibility for the final contents. All authors read and approved the final manuscript.

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peroxisome proliferator-activated receptor gamma (PPAR γ) in white adipose tissue (WAT), decreasing the expression of leptin in WAT and increasing the mRNA expression and protein of glucose transporter 4 (GLUT4) in skeletal muscle [7]. BM extract was able to act on a natural PPARγ signaling pathway in murine hepatoma cell line [8] and increased both glucose uptake and amino acid uptake in L6 cells [9]. A recent study reported that BM extract improved insulin sensitivity by increasing skeletal muscle insulin-stimulated IRS-1 tyrosine phosphorylation in rats fed a high-fat diet [10] and increased hepatic IRS and phosphonositide-3 kinase interactions [11]. However, the precise mechanisms contributing to the *in vivo* actions of BM extract on carbohydrate metabolism, whether via regulation of insulin release or improvement in insulin signaling, remain unknown. Clearly, in-depth studies that combine the clinical effect with mechanistic studies of BM for treating diabetes are needed.

Insulin is the major hormone controlling critical energy functions such as glucose and lipid metabolism. Insulin activates the insulin receptor tyrosine kinase, which phosphorylates and recruits different substrate adaptors including the IRS family of proteins. Tyrosine phosphorylated IRS then displays binding sites for numerous signaling partners. Among them, PI3K has a major role in insulin function mainly via the activation of Akt/PKB. Insulin stimulates glucose uptake in muscle and adipocytes via translocation of GLUT4 vesicles to the plasma membrane [12]. In addition, lipotoxicity (increased tissue fat content) has been implicated in the development of muscle insulin resistance and type 2 diabetes mellitus (T2DM) [13]. It was revealed that reduced adiposity in BM-fed rats is associated with increased lipid oxidative enzyme activities and uncoupling protein expression (UCP) [14].

Thus, based on the proposed *in vivo* effects of BM, we hypothesize that BM mediates an antidiabetic effect and improves glucose metabolism by enhancing insulin sensitivity and altering lipid content in muscle tissues. To test this hypothesis, we conducted a comprehensive study to evaluate mechanisms operative *in vivo* and *in vitro*.

2. Materials and methods

2.1. Materials

BM extract containing the proposed bioactives (aquatic extracted powder) was purchased from IdeaSphere, Inc. (American Fork, UT, USA) and was standardized to contain >8.9% momordicosides A, F2, K and L. HPLC/LC-MS analysis was used to confirm the presence of molecular masses corresponding to each of the momordicosides as well as UV and MS chromatograms that were consistent with another commercially obtained extract of BM. A high-fat diet containing 58% of energy from fat (D-12331) and a low-fat diet containing 10% kcal from fat (D12250B) were purchased from Research Diets, Inc. (New Brunswick, NJ, USA). [1,2-^{3}H(N)] 2-Deoxy-D-glucose (2DG) and [r^{32}P]ATP were purchased from PerkinElmer Life Science (Boston, MA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the Pennington Biomedical Research Center. Twenty male C57BL/6J mice at age 5 weeks were ordered from Charles River Laboratories, Inc. (Wilmington, MA, USA) and were randomly divided into low-fat diet control (LFD as negative control, n=6), high-fat diet group (HFD as positive control, n=7) and a BM extract-treated HFD group (BM, n=7). BM was administrated through food intake by incorporating BM extract into the high-fat diet at 1.2% w/w, based on a previous study [14]. Food intake (the difference of weight administered to the treatment groups and leftover plus spillage) and body weight were recorded weekly. Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IIGTT) were performed at Week 10 and Week 11, respectively. Fasting (4 h) plasma glucose, insulin and body composition were determined at Weeks 0, 6 and 12 of the experimental period. Metabolic chamber experiments to assess energy expenditure were performed at Week 8. At the end of the study, each group was divided into basal and insulin-stimulated subgroups that were matched by body weight. The vastus lateralis muscle was collected at basal condition (n=3, fasting overnight, ~12 h) and insulin-stimulated condition (n=3–4, at 10 min post intraperitoneal injection of insulin at a dose of 2 U/kg body weight). Plasma, skeletal muscle and other tissues were also collected and quickly frozen in liquid nitrogen and stored at −80°C for later analysis.

2.3. Blood chemistry and hormone analysis

After 4 h of fasting, blood samples were collected from the orbital sinus in unconscious mice after a few seconds of CO_2 inhalation. Plasma glucose levels were measured by a colorimetric hexokinase glucose assay (Sigma Diagnostics, St. Louis, MO, USA). Plasma insulin level was determined by an ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kit from Crystal Chem (Downers Grove, IL, USA). Homeostasis model assessment for determining insulin resistance (HOMA-IR) was calculated using the following formula: HOMA=[I_0 (μU/ml)×G_0 (mmol/L)]/22.5 [15]. Plasma leptin was measured as described in our previous study [16].

2.4. Body composition measurement

Body composition for all animals was measured by nuclear magnetic resonance (NMR-Bruker, Newark, DE, USA) [16]. Total fat mass (FM) and free fat mass (FFM) were recorded.

2.5. Metabolic chamber (energy expenditure) study

At Week 8, the mice were placed into Oxymax System metabolic chambers (Columbus Instruments International Corporation, Columbus, OH, USA) with *ad libitum* access to water and the indicated diet for 7 days. Their locomotion, food and water intake, O_2 consumption and CO_2 emission were automatically monitored. The respiratory exchange rate (RER, also known as RQ) was calculated as VCO_2/VO_2.

2.6. Intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test

IPGTT and IPITT were performed at Week 10 and Week 11, respectively, as described in a previous study [16]. Briefly, after overnight fasting, IPGTT was performed by IP injection of 2 g glucose/kg body weight using 20% glucose in 0.9% NaCL or after 4 h of fasting; IPITT was conducted by IP insulin injection of 0.75 U/kg body weight. Blood glucose concentrations were measured from nicked tail vein at Time 0 (baseline), 15, 30, 60 and 120 min after glucose or insulin injections using the Freestyle blood glucose monitoring system (Thera Sense, Phoenix, AZ, USA).

2.7. Western blot analysis

Muscle tissue lysates were prepared by dissection and homogenized in Buffer A [25 mM HEPES, pH 7.4, 1% Nonidet P-40 (NP-40), 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin] using a PRO 200 homogenizer (PRO Scientific, Oxford, CT, USA). The samples were centrifuged at 14,000×g for 20 min at 4°C, and protein concentrations of the supernatant were determined by the Bio-Rad protein assay kit (Bio-Rad laboratories, Inc. Hercules, CA, USA). Supernatants (50 μg) were resolved by SDS-PAGE and subjected to immunoblotting. The protein abundance was detected with antibodies against IRS-1, IRS-2, anti-phospho-IRS-1$^{(Tyr612)}$, p85 of PI 3K, Akt1, Akt2, phosph-Akt1 $^{(ser473)}$ (Upstate, Lake Placid, NY, USA), phosph-Akt2$^{(ser474)}$ (GenScript Co, Piscataway, NJ, USA) and β-actin using chemiluminescence reagent plus from PerkinElmer Life Science (Boston, MA, USA), and quantified via a densitometer. All the proteins were normalized by β-actin, and specific protein phosphorylation was normalized by the corresponding protein shown in the legends.

2.8. PI 3 kinase activity assay

IRS-1-associated PI 3 kinase activities of the muscle at baseline (0 min) and 10 min post insulin stimulation (2 U/kg body weight via intraperitoneally) were assessed as previously described [17]. Briefly, 500 μg of muscle lysates was immunoprecipitated with 3 μg of IRS-1 antibody and protein A agarose. IRS immune complexes were incubated (10 min, 22°C) in 50 μl of 20 mM Tris/HCl (pH 7.0) buffer containing 50 μM [-^{32}P]ATP (5 μCi, Perkin Elmer, Boston, MA, USA), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 100 mM NaCl, 2 mM EDTA and 10 μg of phosphatidylinositol (PI). After thin layer chromatography, radiolabeled phosphatidylinositol 3-phosphate was visualized by autoradiography and quantitated by a densitometer (BioRad, Inc., Hercules, CA, USA).

2.9. Quantitation of muscle acyl carnitines

At 12 weeks, vastus lateralis muscle was collected from the mice, snap frozen and muscle extracts prepared for analysis of fatty acyl carnitine by mass spectrometry at the School of Veterinary Medicine of Louisiana State University as previously described [18].

2.10. Cell culture and BM extract treatment

L6 muscle cells were cultured to myotubes as described by Hajduch et al. [19]. L6 cells were grown in αMEM containing 2% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B) at 37°C with 5% CO_2/95% oxygen. Fully differentiated myotubes were serum starved for 18 h in Dulbecco's Modified Eagle's Medium containing 0.2% bovine serum albumin prior to experiments.

2.11. 2-Deoxy-D-glucose uptake in muscle cells

L6 myotubes in 24-well plates were treated with or without various doses of BM extract (1–10 μg/ml) overnight. After washing with PBS, cells were exposed to 0 or 100 nM insulin in Krebs-Ringer HEPES (KRH) buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$ and 1.3 mM $MgSO_4$) for 15 min followed by an additional incubation period of 5 min with 2DG (100 μM, 0.5 μCi). The cells were washed four times with ice-cold KRH buffer and lysed by adding 250 μl of 0.05N NaOH, then transferred to vials with scintillation cocktail. The radioactivity in the cells was measured by a liquid scintillation counter. Nonspecific uptake was measured using the cells pretreated with 20 μM of cytochalasin B.

2.12. Statistical analysis

All data were expressed as mean±S.E.M. Comparisons of groups were done by *t* test (two-sided) or ANOVA for experiments with more than two subgroups. *P* value <.05 was considered significant.

3. Results

3.1. Effect of BM extract on body weight, food intake, energy expenditure and body composition in mice

At baseline (Week 0), the body weights of the three groups of animals were 20.8±0.98, 21.3±1.02 and 21.2±0.87 g, then increased at Week 12 to 27.1±1.1, 39.9±0.7 and 37.4±0.4 g in LFD, HFD and BM groups, respectively (LFD vs. HFD, *P*<.01; BM vs. HFD, *P*<.05; Fig. 1A). The food intake (normalized by body weight) was significantly higher in both HFD and BM groups than in LFD control mice in the first 3 weeks, but was significantly lower in the BM group than in the HFD group beginning at the first week on diets (Fig. 1B, *P*<.01 and *P*<.05). After 4 weeks of feeding, there was no significant difference among all three groups. Analysis of metabolic parameters demonstrated that the RER was significantly lower in the HFD and the BM groups than in the LFD control group, but there was no difference between the BM and the HFD groups (Fig. 2A and B). The body composition results demonstrated that the HFD feeding resulted in a significantly increased FM accumulation (increase about twofold at Week 6 and threefold at Week 12 in comparison with their baseline). The FM (%) was slightly increased with time in the LFD mice. However, FFM (%) was significantly lower in both the HFD and BM groups than in the LFD group (*P*<.001, Fig. 2C). FFM (%) was slightly higher in the HFD group than in the BM group, but was not felt to be significantly different.

3.2. BM extract improves glucose metabolism and decreases leptin concentration in high-fat diet-fed mice

Fasting plasma glucose and insulin levels as well as HOMA-IR values were significantly increased in the HFD group (Fig. 3A–C). Animals randomized to the HFD and supplemented with BM (BM group) were observed to have significantly lower glucose, insulin and HOMA-IR compared to the HFD group. Furthermore, BM improved glucose tolerance as measured by IPGTT (Fig. 3D). Specifically, total areas under the glucose curve (Glu_{AUC}) were calculated by the trapezoidal rule with IPGTT data and showed that Glu_{AUC} was 866±32, 1181±56 and 998±49 mg/dl in LFD, HFD and BM groups, respectively. The Glu_{AUC} was significantly higher in HFD and BM groups than in the LFD group (*P*<.001 and *P*<.01), but was significantly lower in the BM group when compared to the HFD

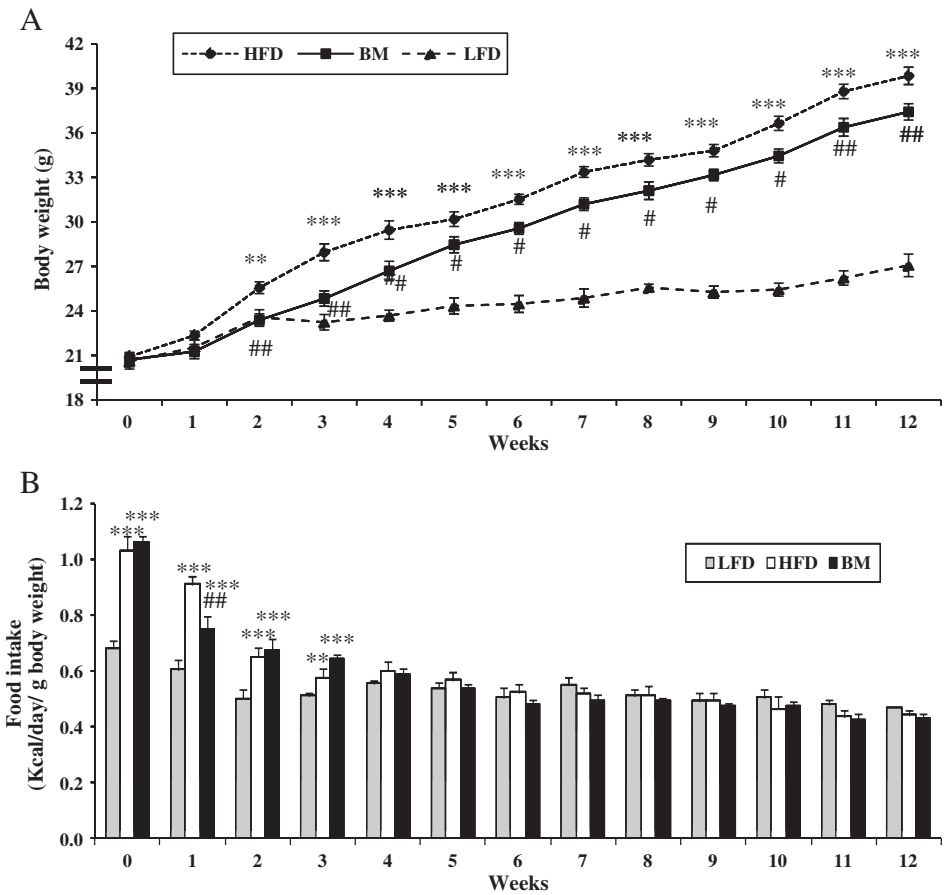

Fig. 1. Effects of BM extract on body weight and food intake in mice. Body weight and food intake were recorded weekly. Panel (A) shows the body weight and Panel (B) illustrates food intake in all three groups of mice. Data are presented as mean±S.E.M. (*n*=6–7/group); *P<.05, **P<.01 and ***P<.001, HFD or BM group vs. LFD control. #P<.05, ##P<.01 BM vs. HFD.

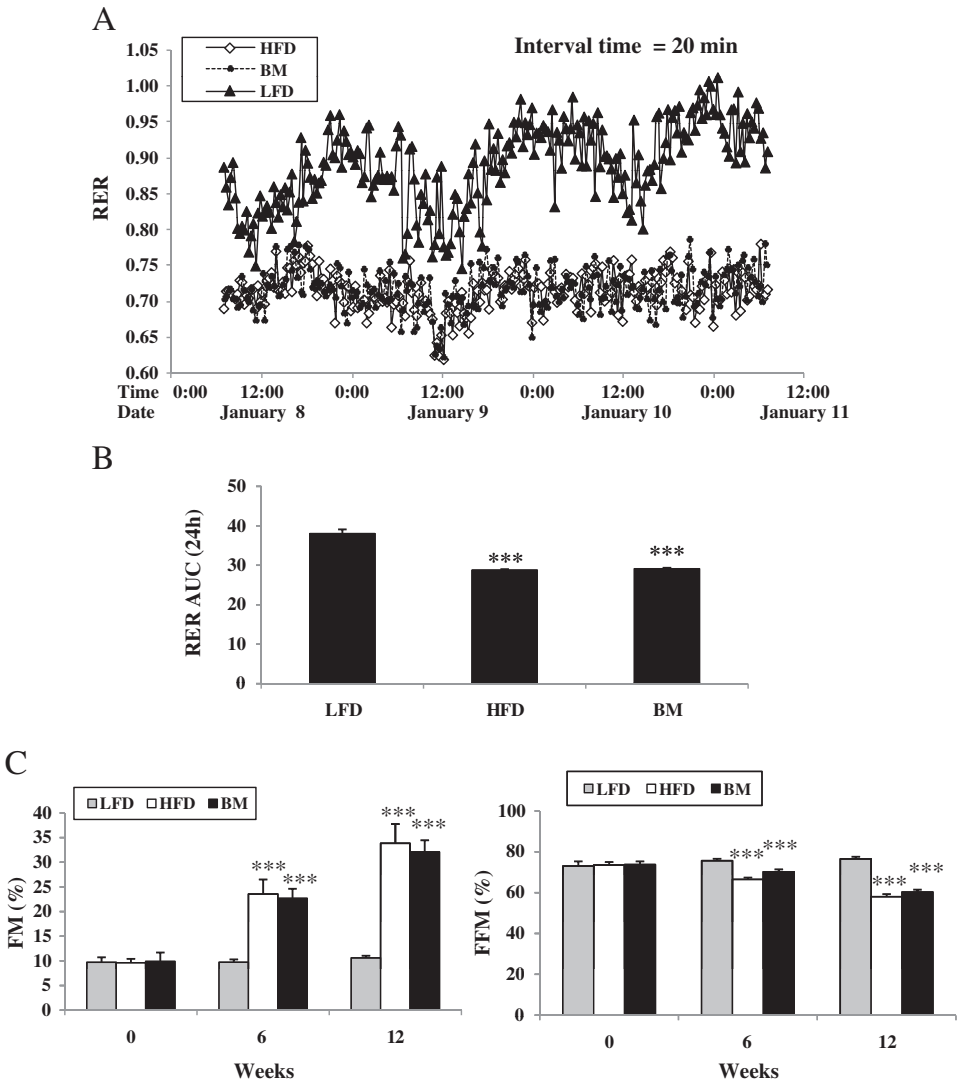

Fig. 2. Energy expenditure and body composition in mice groups. At Week 8, mice were placed into metabolic chambers with water and food for 7 days. Animals' locomotion, O_2 consumption and CO_2 emission were automatically monitored. RER was calculated by VCO_2/VO_2 values and are shown in Panels (A) and (B). Panel (C) shows the body composition measured at Weeks 0, 6 and 12. Fat mass and FFM results are presented as percent of body weight in the three animal groups. Data are presented as mean±S.E.M. (n=6–7/group). **P<.01 and ***P<.001, HFD or BM vs. LFD control. $^{\#}P$<.05, $^{\#\#}P$<.01 BM vs. HFD.

group (P<.01). IPITT data revealed that glucose disposal was the highest in the LFD group among all groups after injection of insulin, and BM extract treatment significantly improved insulin sensitivity when compared with the HFD group (Fig. 3E). At 12 weeks, the plasma leptin concentrations in the HFD and BM groups were significantly higher than in the LFD group (mean±S.E.M. was 8.93±0.6-fold and 7.12±0.36-fold of control, respectively, P<.001), but leptin concentration was approximately 20% lower in the BM group than in the HFD group (P<.01, Fig. 3F).

3.3. Effect of HFD and BM extract on fatty acyl carnitine content in mice muscle tissues

Fatty acyl carnitine profiles were assessed by tandem mass spectrometry in muscle extracts from mice randomized to the LFD, HFD and BM groups. HFD significantly increased both short/medium chain acyl carnitine contents (C3, C4 and C12-OH) and long-chain acyl carnitine contents (C14:2, C14-OH), but decreased C4-DC contents in the muscle when compared with the LFD control. However, HFD supplementation with BM modulated the HFD-induced lipid changes in muscle by significantly reducing fatty acyl carnitine content (Fig. 4A and B).

3.4. BM extract enhances insulin signaling in muscle tissues in the HFD-fed mice

The data of insulin signaling pathway protein analysis demonstrated that IRS-1, IRS-2, PI 3K and GLUT4 protein abundance were significantly lower in HFD than in LFD (P<.05 and P<.001). BM extract supplementation significantly increased IRS-1, IRS-2, PI 3K and GLUT4 plus IR β protein abundance in comparison with the HFD group (Fig. 5A). Insulin-stimulated phosphorylation of IRS1, Akt1 and Akt2 in muscle was significantly lower in the HFD group than in the LFD, but basal phosphorylation of these proteins was not different among the three groups. However, BM supplementation significantly increased basal Akt2 phosphorylation as well as insulin-stimulated phosphorylation of IRS-1, Akt1 and Akt2 when compared with HFD animals (P<.001, P<.05 and P<.01, respectively; Fig. 5B). There were no differences in basal IRS-1 p and Akt 1 p as well as in insulin-stimulated IRS-1 p, Akt 1 p and Akt 2 p between

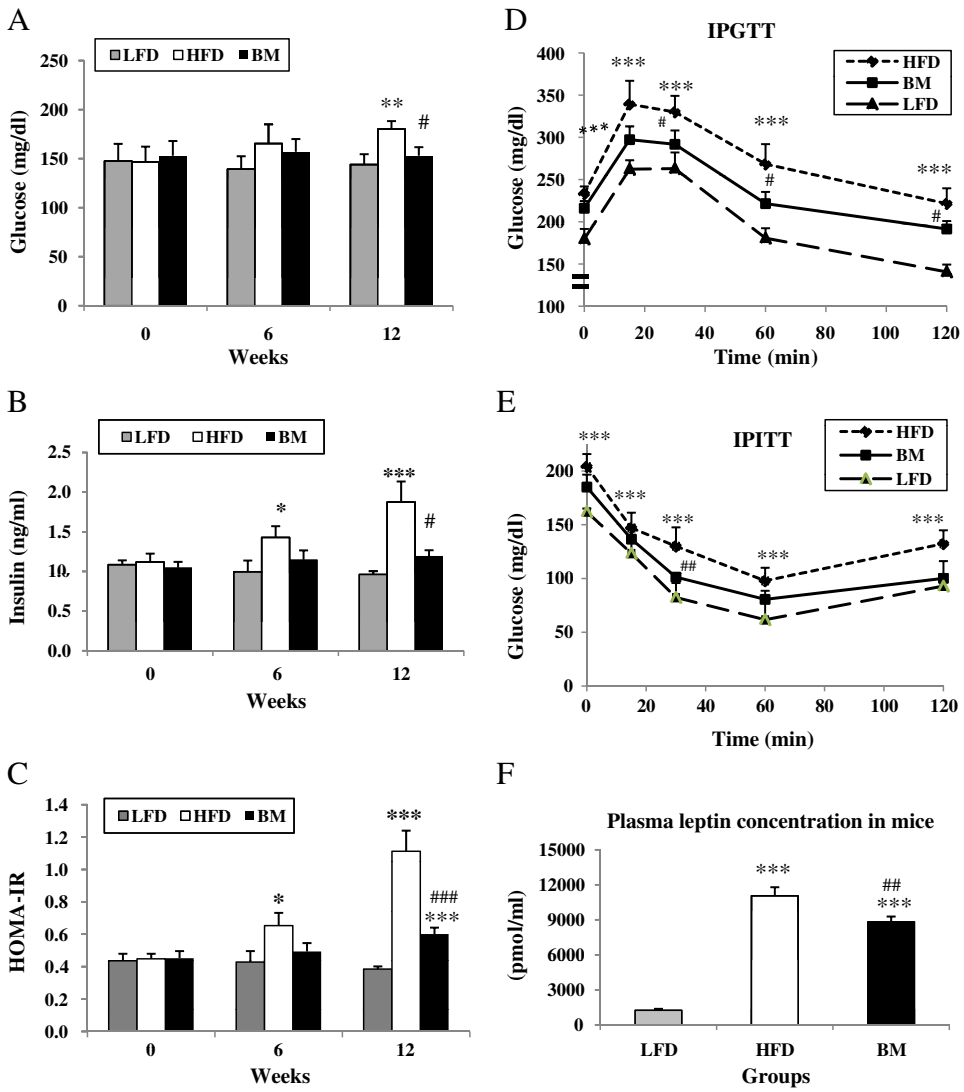

Fig. 3. Glucose, insulin and leptin concentration, and IPGTT and IPITT in mice. Four-hour fasting serum was collected at Weeks 0, 6 and 12, respectively, for measuring glucose and insulin concentration. Panel (A) shows the glucose levels, Panel (B) shows the insulin concentration and Panel (C) shows the HOMA results in the mice. IPGTT was carried out at Week 10 after overnight fasting and IPITT was performed at Week 11 after 4 h of fasting, respectively. These data are shown in Panels (D) and (E). Panel (F) illustrates the plasma leptin concentration at Week 12 in the mice. Data are presented as mean±S.E.M. (n=6–7/group). *P<.05 and ***P<.001, LFD vs. HFD. #P<.05 and ###P<.001, BM vs. HFD.

the LFD and BM groups except that basal Akt 2 p content was significantly higher in BM than in LFD mice. The results of IRS-1-associated PI-3 kinase (PI 3K) activity in muscle revealed that the HFD did not significantly alter basal PI 3K activity, but significantly reduced insulin-stimulated PI3K activation. Moreover, BM supplementation significantly increased basal and insulin-stimulated PI 3K activities by 1.4- and 2.2-fold in comparison with HFD (P<.05 and P<.001, respectively; Fig. 5C).

3.5. Effect of BM extract on glucose uptake and insulin signaling in cultured muscle cells

To test whether BM extract has a direct beneficial effect on improving glucose metabolism and insulin signaling independent of possible confounders, i.e., calorie intake, as would be present in the animal study, we conducted *in vitro* experiments to measure 2DG uptake and insulin signaling in L6 myotubes treated with various doses of BM extract (indicated in the legends of figures). We demonstrated that BM extract treatment dramatically increased both basal and insulin-stimulated glucose uptake (P<.05 and P<.01, respectively; Fig. 6A). BM also substantially increased IR β, IRS-2, PI 3K and GLUT 4 protein abundance in L6 cells at a BM dose of over 2.5 μg/ml (Fig. 6B). Furthermore, BM extract not only significantly increased basal Akt 1 p abundance at a dose of over 5 μg/ml, but also enhanced insulin-stimulated phosphorylation of IRS-1 and Akt1 in a dose-dependent manner (Fig. 6C).

4. Discussion

In this study, we report on the effects of bioactives from BM extract to favorably enhance glucose metabolism and insulin signaling in skeletal muscle tissues from mice fed a high-fat diet. Our results demonstrate that bioactives in BM, when supplemented in a high-fat diet, significantly increased insulin sensitivity *in vivo* as assessed by HOMA index and IPITT. In addition, cellular insulin signaling in skeletal muscle, such as increased IRS-1, IRS-2, IR β, PI 3K and GLUT4 protein abundance and elevated PI 3K activity, was enhanced by BM as assessed in both *in vivo* and *in vitro* studies. Moreover, BM supplementation significantly modulated muscle fatty acyl carnitine content when compared with the HFD group. We also noted no

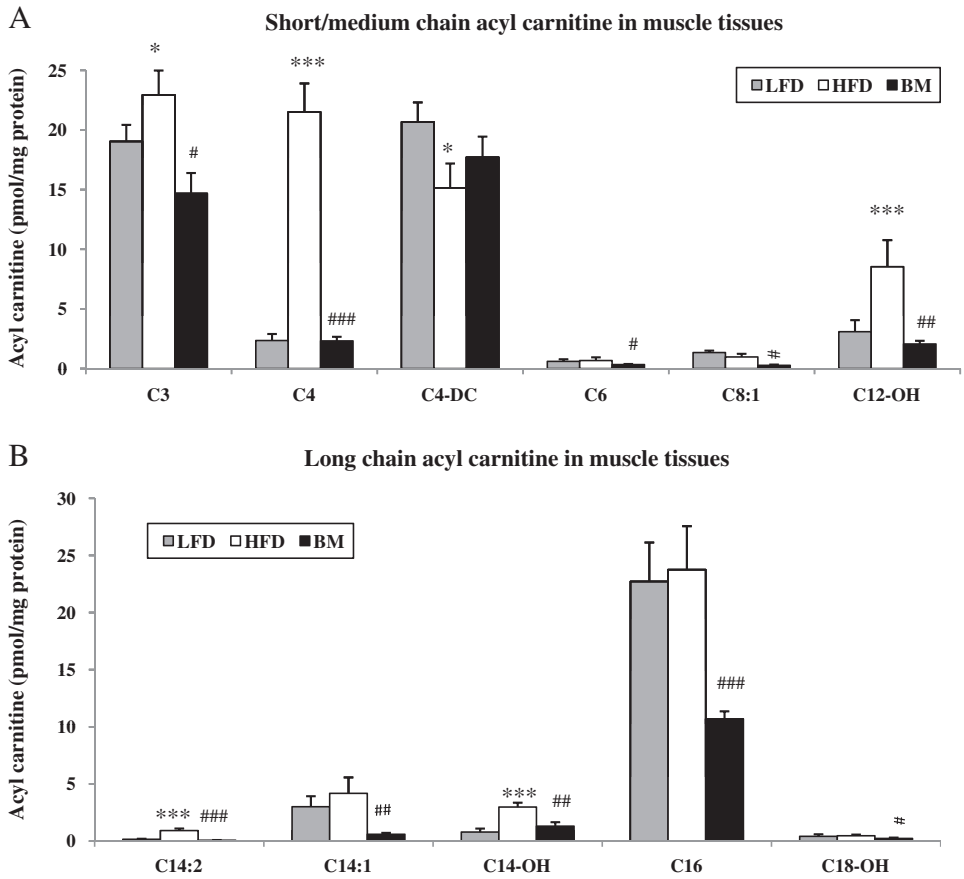

Fig. 4. Fatty acyl carnitine profiles were obtained by tandem mass spectrometry in muscle extracts from mice fed with low-fat diet (n=6, grey bars), high-fat diet (n=7, white bars) and BM extract supplementation (n=7, black bars). The effect of HFD and BM on short/medium chain (Panel A) and long-chain fatty acyl carnitine (Panel B) was assessed by ANOVA as described in Materials and Methods. For each acyl carnitine subtype, C3, acrylylcarnitine; C4, butyrylcarnitine/isobutyrylcarnitine; C4DC, succinyl carnitine; C6, 3-hydroxy-*cis*-5-octenoyl carnitine; C8:1, octanoyl-L-carnitine; C12-DC, dodecenoyl-L-carnitine; C14-2, tetradecadienyl-L-carnitine; C14-OH, tetradecenoyl-L-carnitine; C16, hexadecanoyl-L-carnitine; C18-OH, 3-hydroxysteraroylcarnitine. Mean±S.E.M.; *P<.05, and ***P<.001, HFD or BM vs. LFD. $^{\#}P$<.05 and $^{\#\#}P$<.01, BM vs. HFD.

differences in energy expenditure and body composition between the BM and HFD animals. Consistent with prior reports in rats [14], we observed that the significantly reduced body weight gain of BM mice is not caused by lower energy intake.

The main finding from this study lies in the effects of BM extract on reducing plasma leptin and insulin concentrations as well as on enhancing muscle insulin signaling in the high-fat diet-fed mice. We observed that mice developed insulin and leptin resistance during diet-induced obesity (DIO). Leptin behaves as a potent anorexic and energy-enhancing hormone in most young or lean animals, but its effects are diminished or lacking in the obese state associated with a normal genetic background [20]. In DIO, hypothalamic and systemic inflammatory factors trigger intracellular mechanisms that lead to resistance to the main adipostatic hormones, leptin and insulin [21]. *In vitro* studies have demonstrated the inhibitory effect of leptin on insulin signaling; Morrison et al. [22] recently reported that there is a crosstalk between leptin and insulin signaling during the development of DIO [20,21]. Further evidence has shown that leptin impairs insulin signaling in rat adipocytes [23]. Interestingly, metformin, a hypoglycemic drug, can restore leptin sensitivity in high fat-fed obese rats with leptin resistance [24]. It is established that leptin increases PI 3K activity in liver, leading to lowered hepatic triglyceride levels in lean rats, but not in obese animals on a high-fat diet [25]. Since PI 3K couples leptin and insulin signaling pathways via IRS-1 and IRS-2, it could be argued that a defective activation of PI 3K could be a novel mechanism of peripheral leptin or insulin resistance [26]. Recently, Nerurkar et al. reported that BM extract significantly reduced plasma apolipoprotein B-100 and increased hepatic IRS-1 and PI 3K activity in mice fed a high-fat diet [11]. Our findings support the concept that defective PI 3K activity may also contribute to insulin resistance in skeletal muscle in the HFD-induced obese animals. We demonstrated that HFD results in insulin and leptin resistance in mice with significantly reduced insulin-stimulated PI 3K activity in the muscle. Consistent with the Sridhar et al. [10] study, we observed that BM significantly increased muscle IRS-1 phosphorylation in the mice fed a HFD. Thus, our study suggests that bioactives in the BM extract were able to attenuate the effect of a HFD on the insulin signaling pathway with significant reduction of plasma insulin and leptin levels. BM-mediated reductions in insulin and leptin may partially result from decreasing body weight in mice fed a HFD. Emerging evidence suggests that leptin resistance predisposes the animal to exacerbated DIO and the elevated plasma concentration of leptin in obesity is correlated with adipose tissue mass [20]. Furthermore, we demonstrated that BM directly increases IRS, PI 3K and GLUT4 protein expression in cultured muscle cells, an *in vitro* model and condition for which clinical factors, i.e., calorie intake and body weight, would not be confounding variables (Fig. 6B and C).

Akt1 and Akt2 are the key downstream intermediates within the PI 3K pathway linked to insulin action on GLUT4 in adipocytes and skeletal muscle [24]. Moreover, an Akt2 gene mutation leads to a dominantly inherited syndrome of insulin-resistant diabetes and partial lipodystrophy [27]. Interestingly, the isoforms of Akt, i.e., Akt1 and Akt2, are not functionally redundant. Mouse gene knockout studies, as well as knockdown studies in 3T3L1 adipocytes using small

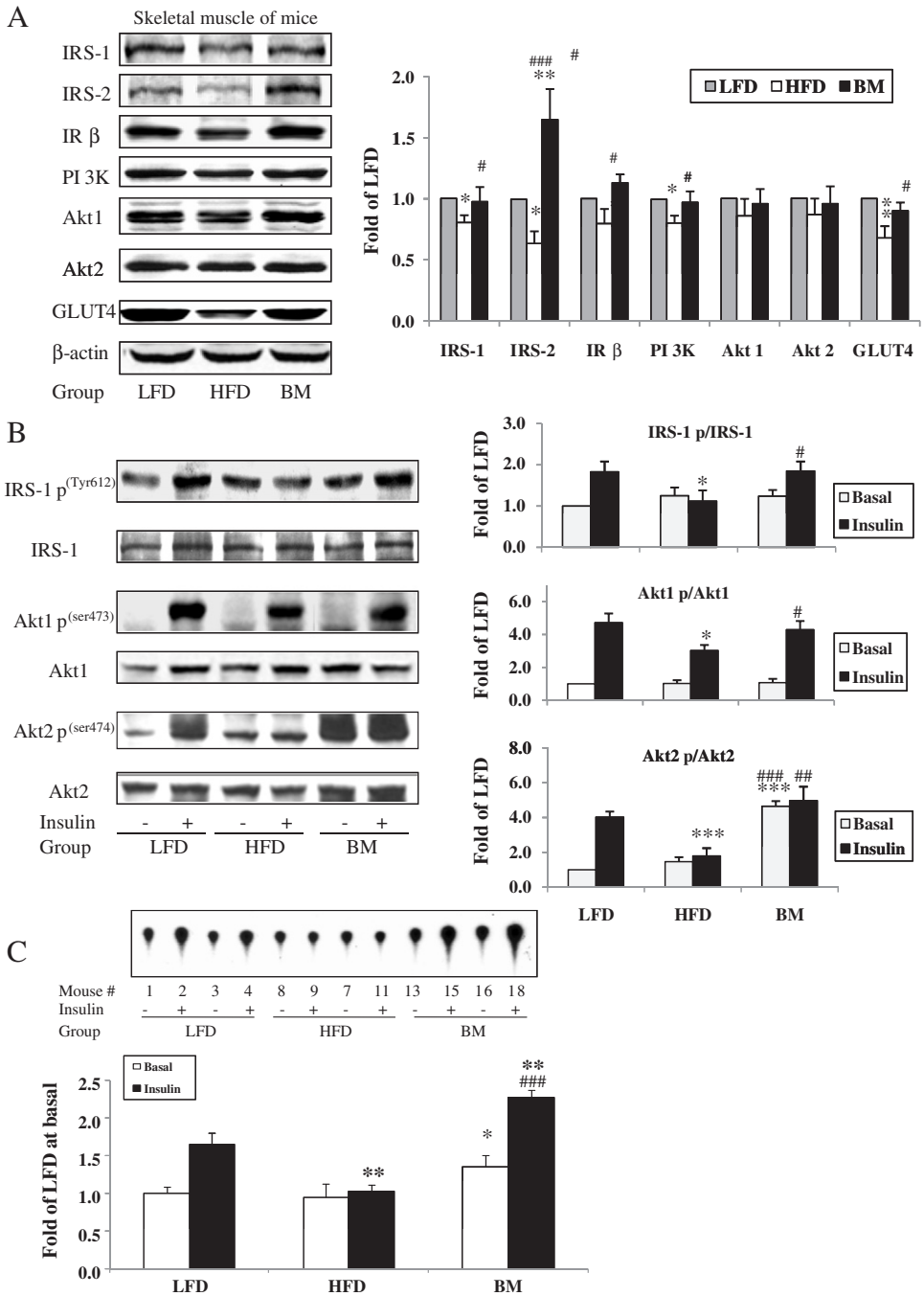

Fig. 5. Effect of BM extract on insulin signaling pathway proteins in mice muscle tissues. IRS-1, IRS-2, IR β, PI3K, Akt1, Akt2 and GLUT4 in the muscle lysates were determined by Western blotting. Panel (A) shows protein abundance in the muscle lysates. Results were normalized by β-actin. Panel B shows the phosphorylation of IRS-1, Akt1 and Akt2 normalized by their corresponding protein levels. Panel (C) shows the muscle PI 3 kinase activity in the mice. Five hundred micrograms of muscle lysate was immunoprecipitated with anti-IRS-1 antibody and protein A agarose. IRS-1-associated PI 3K activity was measured by adding a reaction buffer containing [r^{32}P]ATP, PI and MgCl for 20 min; details are described in Materials and Methods. Data are presented as mean±S.E.M. (n=6–7/group). *P<.05, **P<.01 and ***P<.001, HFD or BM vs. LFD. $^{\#}P$<.05, $^{\#\#}P$<.01 and $^{\#\#\#}P$<.001, BM vs. HFD.

interfering RNA, have shown that reducing only Akt1 does not alter insulin sensitivity, whereas reducing Akt2 levels decreases insulin sensitivity and reduces glucose disposal [27]. In addition, the metabolic phenotype was more profound when both Akt1 and Akt2 protein levels were reduced [28,29]. Therefore, both Akt1 and Akt2 are required for insulin signaling. Direct interaction between PI 3K and membrane vesicles leads to mobilization of GLUT4 glucose transporters in response to insulin stimulation in adipose and muscle cells [30].

Another major finding of our study is that BM may modulate lipid content in skeletal muscle in HFD-fed mice. Evidence has been accumulating that insulin resistance is accompanied by mitochondrial dysfunction in skeletal muscle and liver, and that this could be the proximal cause of impaired lipid oxidation and accumulation of intramyocellular lipid [31–33]. It was believed that incomplete muscle long-chain fatty acid (LCFA) β-oxidation increases the tissue accumulation of acetyl-CoA and generates chain-shortened acyl carnitine molecules implicated in insulin resistance, but molecular

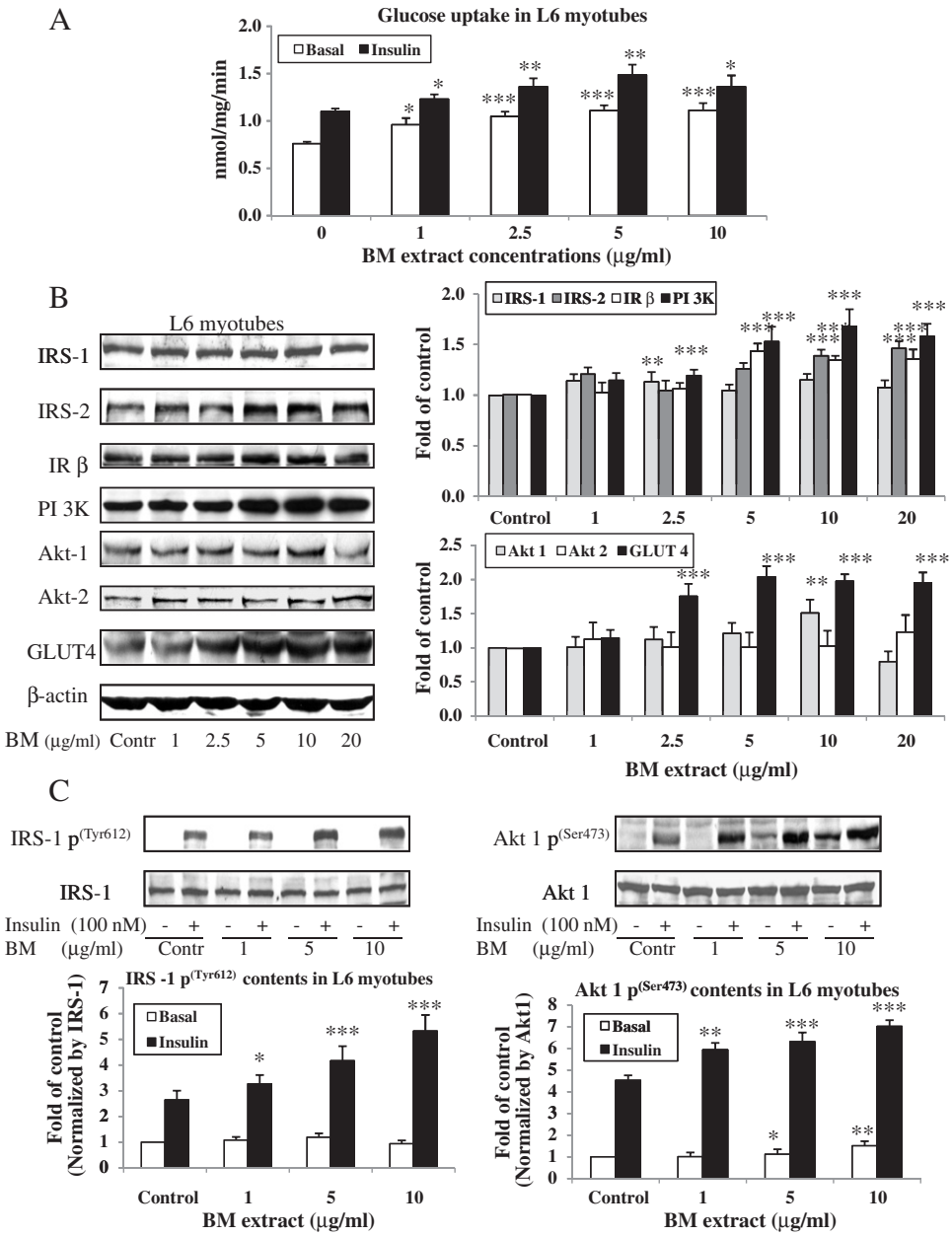

Fig. 6. Glucose uptake and insulin signaling measurements in BM extract-treated L6 myotubes. At Day 6 post differentiation, cells were treated with various doses of BM extract (shown in legends) overnight (16 h). 2-Deoxy-D-glucose uptake was measured with [^{3}H]-2-deoxy-D-glucose assay, and insulin signal transduction pathway protein contents were analyzed by Western blotting. Panel (A) shows the glucose uptake results. Panel (B) shows the insulin signaling protein abundance normalized by β-actin in BM-treated L6 myotubes. Panel (C) illustrates the phosphorylation of IRS-1 and Akt1 normalized by IRS-1 and Akt1 protein levels, respectively. Data are presented as mean±S.E.M. from three independent experiments. *P<.05, **P<.01 and ***P<.001, BM vs. control, respectively.

links between mitochondrial fat catabolism and insulin action remain controversial [34,35]. To determine whether LCFA combustion is associated with insulin resistance, Adams et al. [36] analyzed plasma acyl carnitine profile in BMI- and age-matched overweight to obese type 2 diabetic and non-diabetic African-American women and demonstrated that inefficient tissue LCFA β-oxidation, due in part to a relatively low tricarboxylic acid cycle capacity, increases tissue accumulation of acetyl-CoA and generates chain-shortened acyl carnitine molecules that activate proinflammatory pathways implicated in insulin resistance. Thus, HFD compromised acylcarnitine metabolism with increased skeletal muscle accumulation of acyl carnitine esters and insulin resistance [36,37]. It was reported that reduced acyl carnitine reverses marked perturbation in mitochondrial fuel metabolism including low rates of complete fatty acid oxidation, elevated incomplete β-oxidation and impaired substrate switching from fatty acid to pyruvate in the muscle of obese rats [38]. A recent study showed that pioglitazone improved insulin resistance in T2DM in association with mobilization of fat and toxic lipid metabolites out of muscle [13]. Our study demonstrated that HFD significantly increased fatty acyl carnitine content in skeletal muscle when compared with the LFD mice, while BM supplementation substantially attenuated high-fat diet-induced fatty acyl carnitine accumulation in muscle. Thus, the modulation of lipid metabolism by BM in muscle may be associated with the improved insulin action, but clearly our data do not suggest cause and effect. Our data support a model in which mitochondrial overload and incomplete fatty acid

oxidation contribute to skeletal muscle insulin resistance [39]. Although there was no difference in energy expenditure between HFD and BM groups, we confirmed that BM extract treatment significantly increased fatty acid oxidation and decreased triglyceride content in cultured muscle cells (data not shown) and a similar finding of BM increasing lipid oxidative enzyme activities and UCP expression was observed in a rat study [14]. Therefore, the reduction of muscle lipids by BM may play a role in enhancing insulin signaling.

It was previously reported that incubation of L6 myotubes with different concentrations of BM extract resulted in time-dependent increases in 2DG with maximal uptakes occurring at a concentration of 5 μg/ml [40]. Consistent with this report, we observed a similar optimal concentration of BM in increasing glucose uptake and insulin signaling proteins in L6 myotubes. It was reported that BM extract mimics insulin in its ability to exert a hypoglycemic effect and stimulates amino acid uptake into skeletal muscle cells [9]. We also demonstrated the effect of BM on insulin signaling with significantly increasing basal phosphorylation of Akt1 and Akt2, and PI 3K activity in mice muscle. In addition, BM not only significantly increased basal glucose uptake, PI 3K and GLUT4 protein expression of L6 myotubes in a dose-dependent manner, but also significantly increased basal Akt 1 phosphorylation, beyond its insulin synergy. Therefore, our study suggests that the effect of BM on improving glucose metabolism and enhancing insulin signaling may mimic the action of insulin. Nevertheless, the effect of BM on insulin signaling in cultured cells may not precisely mirror the mechanism or response seen *in vivo*. Clearly, the difference between the *in vivo* and *in vitro* studies may be due to the variance of BM dosages or to its absorptive bioactive components in the animals.

The limitation of the current study is that we are unable to determine whether BM extract affects processes via actions in the central nervous system or whether BM extract alters secretion of gut hormones. Another limitation is that, at this time, we are not able to specifically measure the proposed bioactives in plasma and there is limited data currently on bioavailability and plasma appearance of bioactives after ingestion.

5. Conclusion

Bitter melon extract significantly reduced body weight, improved glucose metabolism and enhanced insulin signaling in mice fed with HFD. In addition, BM modulated acylcarnitine patterns in muscle observed with HFD feeding. As such, nutritional supplementation with this extract, if validated in human studies, may provide an adjunctive therapy for the treatment of obesity and diet-induced insulin resistance.

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References

[1] Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature 2001;414:782–7.
[2] Reaven GM. Role of insulin resistance in human disease. Diabetes 1998;37:1595–607.
[3] Hill JO, Wyatt HR, Reed GW, Peters JC. Obesity and the environment: where do we go from here? Science 2003;299:853–5.
[4] Basch E, Gabardi S, Ulbricht C. Bitter melon (*Momordica charantia*): a review of efficacy and safety. Am J Health-Syst Pharm 2003;60:356–9.
[5] McCarty MF. Does bitter melon contain an activator of AMP-activated kinase? Med Hypotheses 2004;63:340–3.
[6] Krawinkel MB, Keding GB. Bitter gourd (*Momordica charantia*): a dietary approach to hyperglycemia. Nutr Rev 2006;64(7 Pt 1):331–7.
[7] Shih CC, Lin CH, Lin WL, Wu JB. Momordica charantia extract on insulin resistance and the skeletal muscle GLUT4 protein in fructose-fed rats. J Ethnopharmacol 2009;123(1):82–90.
[8] Chao CY, Huang CJ. Bitter gourd (*Momordica charantia*) extract activates peroxisome proliferator-activated receptors and upregulates the expression of the acyl CoA oxidase gene in H4IIEC3 hepatoma cells. J Biomed Sci 2003;10(6 Pt 2):782–91.
[9] Cummings E, Hundal HS, Wackerhage H, Hope M, Belle M, Adeghate E, et al. Momordica charantia fruit juice stimulates glucose and amino acid uptakes in L6 myotubes. Mol Cell Biochem 2004;261(1-2):99–104.
[10] Sridhar MG, Vinayagamoorthi R, Arul Suyambunathan V, Bobby Z, Selvaraj N. Bitter gourd (*Momordica charantia*) improves insulin sensitivity by increasing skeletal muscle insulin-stimulated IRS-1 tyrosine phosphorylation in high-fat-fed rats. Br J Nutr 2008;99(4):806–12.
[11] Nerurkar PV, Lee YK, Motosue M, Adeli K, Nerukar VR. *Momordica charantia* (bitter melon) reduces plasma apolipoprotein B-100 and increases hepatic insulin receptor substrate and phosphonositide-3 kinase interactions. Br J Nutr 2008;100:751–9.
[12] Zaid H, Antonescu CN, Randhawa VK, Klip A. Insulin action on glucose transporters through molecular switches, tracks and tethers. Biochem J 2008;413(2):201–15.
[13] Bajaj M, Baig R, Suraamornkul S, Hardies LJ, Coletta DK, Cline GW, et al. Effects of pioglitazone on intramyocellular fat metabolism in patients with type 2 diabetes mellitus. Clin Endocrinol Metab 2010 [Epub ahead of print].
[14] Chan LLY, Chen Q, Go AGG, Lam EKY, Li ETS. Reduced adiposity in bitter melon (*Momordica charantia*)-fed rats is associated with increased lipid oxidative enzyme activities and uncoupling protein expression. J Nutr 2005;135:2517–23.
[15] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.
[16] Wang ZQ, Zuberi AR, Zhang XH, Macgowan J, Qin J, Ye X, et al. Effects of dietary fibers on weight gain, carbohydrate metabolism, and gastric ghrelin gene expression in mice fed a high-fat diet. Metabolism 2007;56:1635–42.
[17] Wang ZQ, Zhang XH, Russell JC, Hulver M, Cefalu WT. Chromium picolinate enhances skeletal muscle cellular insulin signaling in vivo obese, insulin-resistant JCR:LA-cp rats. J Nutr 2006;136:415–20.
[18] Stewart LK, Wang Z, Ribnicky D, Soileau JL, Cefalu WT, Gettys TW. Failure of dietary quercetin to alter the temporal progression of insulin resistance among tissues of C57BL/6J mice during the development of diet-induced obesity. Diabetologia 2009;52(3):514–23.
[19] Hajduch E, Alessi DR, Hemmings BA, Hundal HS. Constitutive activation of protein kinase Bα (PKBα) by membrane targeting promotes glucose and system A amino acid transport, protein synthesis and GSK3 inactivation in L6 muscle cells. Diabetes 1998;47:1006–13.
[20] Scarpace PJ, Zhang Y. Leptin resistance: a predisposing factor for diet-induced obesity. Am J Physiol Regul Integr Comp Physiol 2009;296:R493–500.
[21] Zimmet P, Hodge A, Nicolson M, Staten M, deCourten M, Moore J, et al. Serum leptin concentration, obesity, and insulin resistance in Western Samoans: cross sectional study. Br Med J 1996;313:965–9.
[22] Morrison CD, Huypens P, Stewart LK, Gettys TW. Implications of crosstalk between leptin and insulin signaling during the development of diet induced obesity. Biochim Biophys Acta 2009;1792(5):409–16.
[23] Perez C, Fernandez-Galaz C, Fernandez-Agullo T, Arribas C, Andres A, Ros M, et al. Leptin impairs insulin signaling in rat adipocytes. Diabetes 2004;53:347–53.
[24] Kim YW, Kim JY, Park YH, Park SY, Won KC, Choi KH, et al. Metformin restores leptin sensitivity in high-fat-fed obese rats with leptin resistance. Diabetes 2006;55:716–24.
[25] Huang W, dedousis N, Bhatt BA, O'Doherty RM. Impaired activation of phosphatidylinositol 3-kinase by leptin is a novel mechanism of hepatic leptin resistance in diet-induced obesity. J Biol Chem 2004;279:21695–700.
[26] Zhou QL, Park JG, Jiang ZY, Holik JJ, Mitra P, Semiz S, et al. Analysis of insulin signalling by RNAi-based gene silencing. Biochem Soc Trans 2004;32:817–21.
[27] Tan K, Kimber WA, Luan J, Soos MA, Semple RK, Wareham NJ, et al. Analysis of genetic variation in Akt2/PKB-β in severe insulin resistance, lipodystrophy, type 2 diabetes, and related metabolic phenotypes. Diabetes 2007;56:714–9.
[28] Scheid MP, Marignani PA, Woodgett JR. Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. Mol Cell Biol 2002;22:6247–60.
[29] Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc Natl Acad Sci U S A 2003;100:7569–74.
[30] Holman GD, Kasuga M. From receptor to transporter: insulin signaling to glucose transport. Diabetologia 1997;40:991–1003.
[31] Kelley DE, He J, Menshikova EV, et al. Dysfunction in mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 2002;51:2944–50.
[32] Petersen KF, Dufour S, Befroy D, et al. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 2004;350:664–71.
[33] Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest 2008;118:829–38.
[34] Kraegen EW, Clark PW, Jenkins AB, Daley EA, Chisholm DJ, Storlien LH. Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. Diabetes 1991;40:1397–403.

[35] Shulman GI. Cellular mechanisms of insulin resistance. J Clin Invest 2000;106: 171–6.
[36] Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, et al. Plasma acyl carnitine profiles suggest incomplete fatty acid β-oxidation and altered tricarboxylic cycle activity in type 2 diabetic African-American women. J Nutr 2009;139(6):1073–81.
[37] Su X, Han X, Mancuso DJ, Abendschein DR, Gross RW. Accumulation of long-chain acyl carnitine and 3-hydroxy acyl carnitine molecular species in diabetic myocardium: identification of alterations in mitochondrial fatty acid processing in diabetic myocardium by shotgun lipidomics. Biochemistry 2005;44(13):5234–45.
[38] Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolism control. J Biol Chem 2009;284:22840–52.
[39] Koves TR, Ussher JR, slentz D, Mosedale M, Ilkayeva O, Bain JR, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metabolism 2008;7:45–56.
[40] Ahmed I, Adeghate E, Cummings E, Sharma AK, Singh J. Beneficial effects and mechanism of action of *Momordica charantia* juice in the treatment of streptozotocin-induced diabetes mellitus in rat. Mol Cell Biochem 2004;261 (1-2):63–70.