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Full Length Research Paper

Dendrobium moniliforme extract regulates glucose and lipid metabolism through activation of peroxisome proliferator-activated receptor α

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Peroxisome proliferator-activated receptors (PPARs) have been considered as desirable targets for curing metabolic diseases, even though their specific agonists have several side effects. In this study, we demonstrated a novel pharmacological property of methanol extract of *Dendrobium moniliforme* (DM) on metabolic disorders both *in vitro* and *in vivo*. DM extract and its ethyl acetate (EA) fraction selectively stimulated the transcriptional activity of PPARα and regulated expression of genes involved in lipid metabolism in HepG2 hepatocytes, which led to increased mitochondrial and/or peroxisomal fatty acid β-oxidation. Furthermore, the DM treatment significantly decreased body weight gain and feed efficiency in high-fat diet (HFD) mouse model, which were associated with a significant reduction of total cholesterol and free fatty acid levels in the serum. In addition, DM treatment significantly reduced serum levels of both alanine aminotransferase and aspartate aminotransferase compared to the HFD-induced obese mice. Altogether, our findings strongly suggest that DM and its EA **fraction can improve glucose and lipid impairment in HFD mice model and might be considered as a therapeutic agent that is effective for improving glucose and lipid homeostasis without severe side effects.**

Key words: Peroxisome proliferator-activated receptor (PPARα), *Dendrobium moniliforme*, obesity.

INTRODUCTION

Obesity can trigger most of the symptoms of metabolic syndrome, including an increased risk of insulin resistance, nonalcoholic fatty liver, atherosclerosis, degenerative disorders, immune-mediated disorders, and cancers (Gross and Staels, 2007; Henke, 2004; Moller, 2001; Tenenbaum et al., 2003). Dysregulation of fatty acid metabolism in energy balance is a key event responsible for obesity and obesity related diseases (Ronnett et al., 2006). It has been known that peroxisome proliferator-activated receptors (PPARs), members of nuclear hormone receptors, play key roles in the regulation of lipid metabolism, glucose homeostasis and inflammatory processes. Agonists of PPARs are effective drug to improve the metabolic abnormalities linking hyperlipidemia to diabetes, hyperglycemia, insulin resistance, and atherosclerosis (Evans et al., 2004; Ferré, 2004; Kersten et al., 2000; Staels and Fruchart, 2005). The PPAR family consists of 3 isoforms, PPARα, PPARβ/δ, and PPARγ (Lee et al., 2003). Among them, PPARα is expressed predominantly in the liver and serves as important lipid sensors. PPARα-mediated regulation of lipid homeostasis occurs in mitochondria

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and peroxisomes of liver, and PPARα target genes have been mainly concentrated on hepatocytes (Desvergne and Wahli, 1999; Guri et al., 2006). PPARα activation led upregulation of CPT1 (carnitine palmitoyltransferase 1), MCAD (medium-chain acyl-CoA dehydrogenase), ACO2 (acyl-CoA 2), and ECH1 (enoyl-CoA hydrotase 1) gene expression involved in mitochondrial and preoxisomal fatty acid uptake and oxidation (Blaak, 2003; Reddy and Rao, 2006; Roden, 2005). PPARα agonists such as fibrates have been used for treatment dyslipidemia and reducing cardiovascular disease. However, natural PPARα ligands that specifically improve impaired fatty acid metabolism have not yet been identified.

The *Dendrobium* species, a well-known Chinese herbal medicine, distributed in most Asian countries, Europe, and Australia, belongs to the Family Orchidaceae (Ye and Zhao, 2002). Previous chemical and biological investigation of *Dendrobium moniliforme* (Orchidaceae, DM) led to the identification of memory impairment preventing alkaloids (Li et al., 2011), anti-neuro inflammatory alkaloids (Kierkegaard and Pilotti, 1970), immunoregulatory sesquiterpenoids and polysaccharides (Zhao et al., 2001, 1994), antitumor phenanthrenes (Lee et al., 1995; Sanchez-Duffhues et al., 2009), antioxidant alkyl ferulates (Lo et al., 2004), protein tyrosine phosphatase inhibitory phenanthrenes (Bae et al., 2004) and a series of aromatic compounds with anti-platelet aggregation effects (Fan et al., 2001). In addition, the important Chinese herb “Shi-Hu” is prepared from the dried stems of *Dendrobium* species and is used as a tonic and an antipyretic (Jiangsu New Medical College, 1986). However, little is known about anti-obesity and hypolipidemic activity of DM.

In this study, we explored the pharmacological properties of DM on glucose and lipid metabolism. Biochemical analyses revealed that DM is able to directly activate PPARα, which leads to amelioration of glucose and lipid abnormalities. In addition, in high-fat diet (HFD) model, DM improved the abnormalities of lipid metabolism without hepatotoxicity and reduced body weight gain. Altogether, these data suggest that DM would be potential therapeutic agents for treatment of glucose and lipid dysregulation through its PPARα activation potential.

MATERIALS AND METHODS

Plant materials and extraction

The air-dried stems of DM were purchased from Chonnam Herb Association in Korea. The plant was authenticated and deposited at the KIST Gangneung Institute Herbarium, Gangneung, Korea (Voucher No. KIST-164).

DM (600 g) was extracted three times with methanol using ultrasonic apparatus and evaporated under a vacuum at 40°C. The extract (21 g) was reconstituted with 1.0 L of water and was partitioned with *n*-hexane (Hx), methylene chloride (MC), ethyl acetate (EtOAc), and *n*-butanol (BuOH) successively. Each fraction was evaporated under a vacuum at 40°C to obtain Hx (3.4 g), MC (5.6 g), EtOAc (3 g), BuOH (6.7 g), and Water (DW, 3 g) fractions.

Cell culture

CV-1 cells were purchased from the American Type Culture Collection, and cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone Laboratories, Logan, UT) at 37°C with 5% CO_2 in air. HepG2 cells were purchased from the American Type Culture Collection, and cultured in Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS), supplemented with 10% fetal bovine serum (Hyclone Laboratories), 1% penicillin/ streptomycin (Hyclone Laboratories), 1X non-essential amino acid (WelGENE, Daegu, Korea) and 1 mM sodium pyruvate (WelGENE) at 37°C with 5% CO_2 in air.

Luciferase reporter gene assay

CV-1 cells were seeded into 24-well plates and cultured for 24 h before transfection. CV-1 cells were grown in DMEM containing 10% charcoal dextran treated fetal bovine serum (CDT-FBS) and 1% antibiotics. Twenty four hours later, a DNA mixture containing PPRE-luciferase reporter plasmid (0.3 μg), pcDNA3-hPPAR-α/-γ/-δ (30 ng) and internal control plasmid pRL-SV-40 (5 ng) was transfected using TransFast™ transfection reagent (Promega, Madison, WI). After 24 h of transfection, the cells were incubated for an additional 24 h following treatment with positive control (WY14643 for PPARα, troglitazone for PPARγ, and GW501516 for PPARδ, respectively) or the indicated concentrations of DM and its fractions. The luciferase activity of the cell lysates was measured using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI), according to the manufacturer’s instructions. Relative luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity.

Total RNA isolation and real time quantitative PCR

HepG2 cells were seeded into 6-well plates and cultured for 24 h before transfection. HepG2 cells were grown in MEM/EBSS containing 10% CDT-FBS, 1% antibiotics, 1X non-essential amino acid and 1 mM sodium pyruvate. Twenty four hours later, pcDNA3-hPPARα (1 μg) was transfected using TransFast™ transfection reagent (Promega). After 24 h of transfection, the cells were incubated for an additional 48 h following treatment with positive control or the indicated concentrations of DM and its fractions.

Then, total RNA was isolated from HepG2 cells using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The RNA concentration of each sample was determined by spectrophotometer at 260 nm. The integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, SantaClara, CA). cDNA synthesis was performed with 1 μg of total RNA in 20 μl using random primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Real time quantitative PCR (qPCR) analyses for the genes described in Table 1 were performed using the 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA). Reactions were performed in a 25 μl volume containing 12.5 μl of 2 X SYBR Green reaction buffers, 1 μl of cDNA (corresponding to 25 ng of reverse transcribed total RNA) and 5 pmol of each primer. After an initial incubation for 2 min at 50°C, the cDNA was denatured at 95°C for 10 min followed by 40 cycles of PCR (95°C, 15s, 60°C, 60s). Data analyses were performed on 7500 System SDS software version 1.3.1 (Applied Biosystem). All the samples were normalized by the corresponding expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Table 1. Primers used in this study (For Real time PCR).

Gene name		Oligonucleotide Sequence(5'→ 3')	Accession No.
CPT-1	Forward	TCCTGCTTTACAGGCGCAAA	NM_001876.1
	Reverse	CACTGAGCGGAGCAGAGTGG	
MCAD	Forward	TGGGCCAGCGATGTTCAGAT	M16827
	Reverse	TGAAACCAGCTCCGTCACCAA	
ACO2	Forward	GATACACAGCCCCACGCTGA	NM_003500
	Reverse	GCTGACCGTCCCAAGTCTCC	
ECH1	Forward	CTGGCCCTGGGCTGTCACTA	NM_001966
	Reverse	TGCACCAGGGAGAAGTCCCA	
GAPDH	Forward	TGCCACCCAGAAGACTGTGG	NM_002046
	Reverse	AGCTTCCCGTTCAGCTCAGG	

Animal studies

All experiments were performed according to the procedures approved by Korea Institute of Science and Technology's Institutional Animal Care and Use Committee (Certification No. KIST-2011-10-024). Twenty eight mice (7 weeks old male C57BL/6) were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan) and divided into 4 groups (7 mice each groups). The mice were housed under temperature (23±2℃) and humidity (55±5%) condition with a standard light (12/12-h light-dark cycle), and given free access to water and normal diet for a period of one week after arrival. Then mice were fed a regular diet (10% kcal fat, 38057, Purina Inc., Seongnam, Korea) or a HFD (60% kcal fat, D12492, Research Diets, New Brunswick, NJ) for 9 weeks. The mice were orally administered once a day with 500 mg/kg metformin (MT) or 200 mg/kg DM for 9 weeks. During the experimental period, their body weight, food intake and water intakes were measured daily. Weight gain was an increase in body weight during the experimental period, and feed efficiency was calculated as body weight gain divided by food intake. At the end of the experimental period, blood samples were taken from the abdominal aorta to determine the plasma biomarkers.

Analysis of plasma biomarkers

After the experiment, the mice were fasted 16 h and measured fasting blood glucose levels by tail vein bleeds using Accu-Chek glucometer (Roche, Mannheim, Germany). And then, the blood was collected in 0.18 M EDTA containing tubes and centrifuged at 5000 rpm for 5 min at 4℃. After centrifugation, plasma was separated for estimation of total cholesterol, free fatty acid (NEFA), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Total cholesterol levels were measured by enzymatic methods using SICDIA L T-CHO reagents (Eiken Chemical, Tokyo, Japan) and free fatty acid levels were determined by enzymatic methods using SICDIA NEFAZYME (Eiken Chemical). ALT and AST levels were measured by modified IFCC methods using_ALT IFCC reagent and AST IFCC reagent (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Data are expressed as mean ± SD. Differences between the mean values in the two groups were analyzed using Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

The effects of *D. moniliforme* and its fractions on PPARα/γ/δ transcriptional activities

We examined the effects of DM on PPARα/γ/δ transcriptional activities. As shown in Figure 1A, the DM treatment led to a significant increase in PPARα reporter gene activities; however the effect of DM on transcriptional activation of PPARγ or PPARδ was weaker than its effect on PPARα transcriptional activation. We next prepared five fractions (MC, Hx, EA, BuOH, and DW) from DM extract under standard solvent partition, and explored the effect of the fractions on PPARα/γ/δ activation. As shown in Figure 1B, three fractions (MC, Hx, and EA) significantly increased PPARα transcriptional activity. However, the fractions did not affect the transcriptional activity of either PPARγ or PPARδ although the only activation of PPARγ was observed in the presence of MC fraction (Figure 1B). These results strongly indicate the possible presence of specific agonist of PPARα in the extract of DM.

The DM modulates the levels of gene expression for lipid metabolism in HepG2 cells

To explore the potential effect of DM and its fractions on fatty acid metabolism, PPARα-transfected HepG2 cells were treated with DM or its fractions. We examined mRNA expression levels of genes involved in mitochondrial and peroxisomal fatty acid β-oxidation. Treatment with DM increased the mRNA expression of

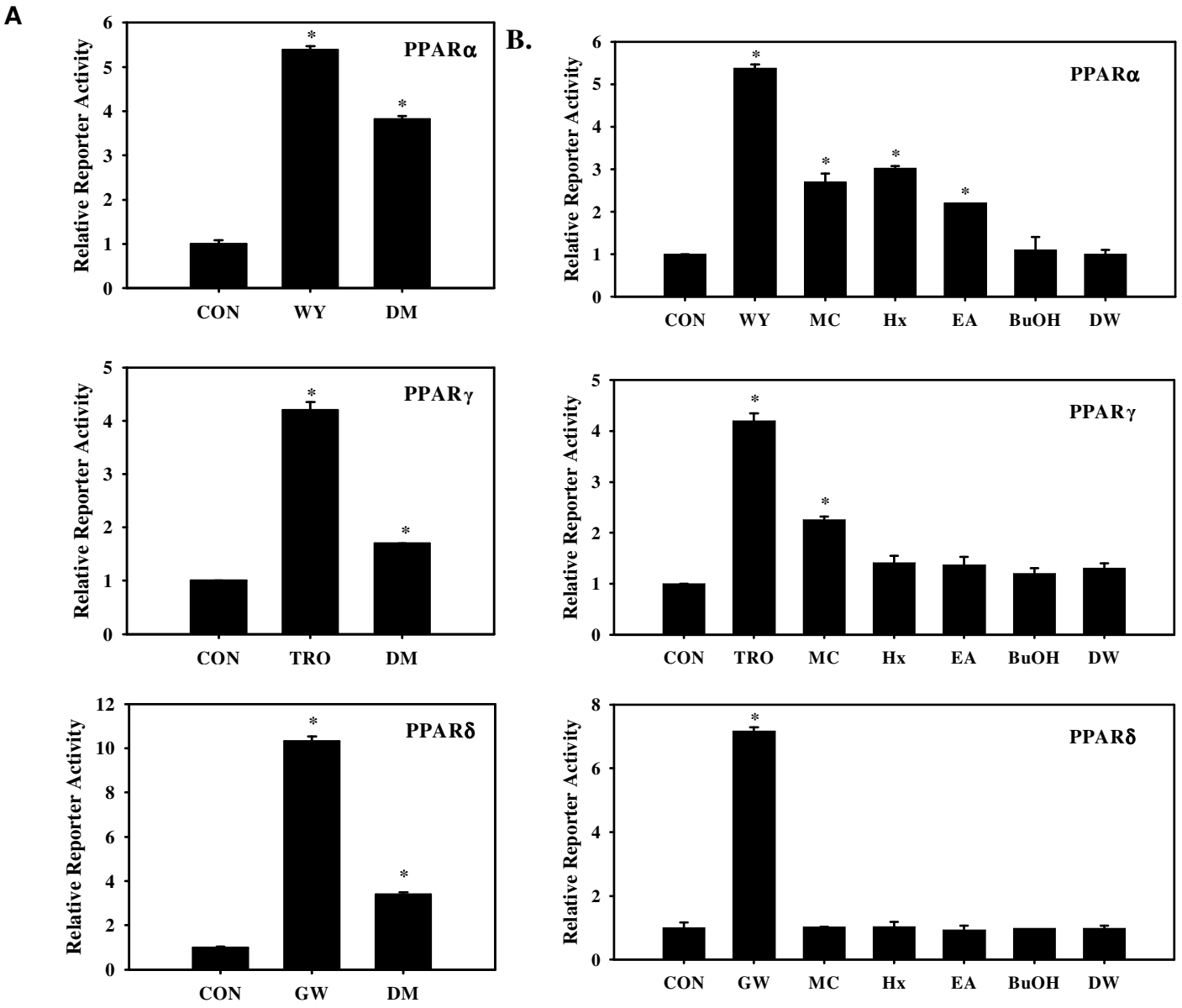

Figure 1. The effects of *Dendrobium moniliforme* extract (A) and five fractions (B) on PPAR α/γ/δ transactivation. CV-1 cells were transfected with a DNA mixture containing PPRE-luciferase reporter plasmid, pCDNA3-hPPAR-α/-γ/-δ and pRL-SV40 for 24 h. The cells were incubated for an additional 24 h following treatment with vehicle (CON), 10 μM WY14643 (WY), 10 μM troglitazone (TRO), 1 μM GW501516 (GW), or 100 μg/ml DM (A), and five fractions (30 μg/ml) (B). Specific agonists for PPAR α/γ/δ were used as positive controls (WY, TRO and GW, respectively). The harvested cells were analyzed by reporter assay as described previouslty. Each bar represents the mean ± SD of duplicates. *P<0.05 vs. control.

both CPT1 and MCAD involved in mitochondrial fatty acid β-oxidation in HepG2 cells, but treatment with MC, BuOH, or DW fraction led to an increase in CPT1 mRNA expression only (Figure 2A and B). Similarly, mRNA expressions of both ACO2 and ECH1 which involve in peroxisomal fatty acid β-oxidation were significantly increased in the presence of DM or EA fraction in HepG2 cells (Figure 2C and D). These findings imply that some components of EA fraction from DM promote peroxisomal fatty acid β-oxidation through the activation of PPARα.

DM treatment significantly blocks body weight gain in HFD mice

To confirm the anti-obesity effects of DM, we used the HFD mouse model and checked the effects on body weight, weight gain and feed efficiency (Figure 3A to C). The body weight, weight gains, and feed efficiency were significantly increased in the group of HFD-fed mice compared to regular diet-fed group. The body weight of DM treated mice was significantly attenuated compared

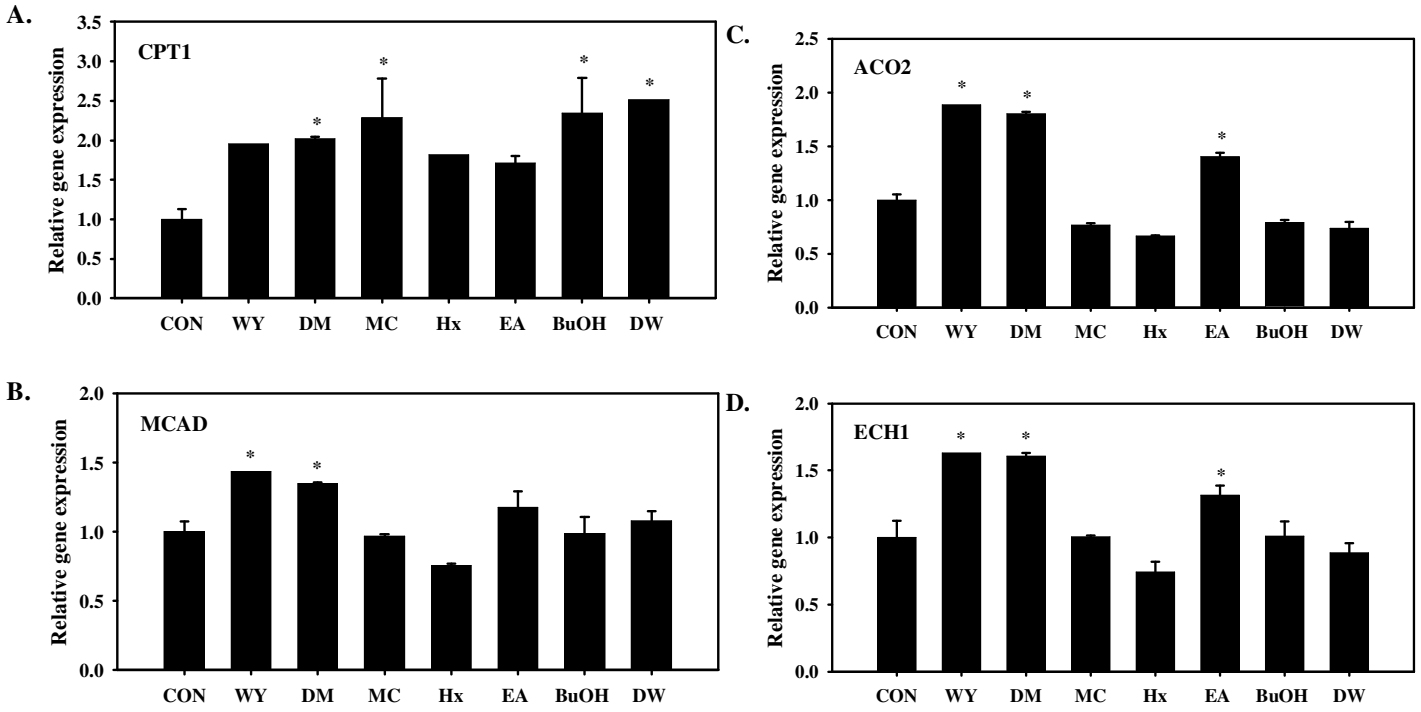

Figure 2. qRT-PCR of genes involved in mitochondrial (A, B) and peroxisomal (C, D) fatty acid metabolism. HepG2 cells were transfected with pCDNA3-hPPARα for 24h, and treated with vehicle (CON), 10 μM WY14643 (WY), μg/ml DM, or 10 μg/ml five solvent fractions (MC, Hx, EA, BuOH, or DW). Gene expression levels of CPT1 (A) and MCAD (B), ACO2 (C), and ECH1 (D) involved in fatty acid β-oxidation in HepG2 cells were quantified by quantitative real time PCR. The mRNA levels were expressed as the fold increase relative to the control after normalization by the GAPDH mRNA expression. Each bar represents the mean ± SD of duplicates. *P<0.05 vs. control.

to the HFD group from 3 week after administration of DM (Figure 3A). Weight gains for 9 weeks in MT and DM treated group were decreased by 59.1 and 22.1%, respectively (Figure 3B). Especially, feed efficiency in MT and DM treated group was significantly decreased by 54.0 and 24.5% respectively compared to HFD group (Figure 3C). The total food intake was same in HFD and DM group, but reduced 11% in MT treated group (data not shown). All of these data support that an anti-obesity effect of DM is mediated, in part, by modulating energy expenditure in HFD-fed mice.

DM can improve plasma glucose and lipid profiles in HFD mice

To confirm anti-diabetic effect of DM, we examined the fasting blood glucose levels in HFD mice model. Treatment of HFD mice with MT and DM led to a dramatic decrease in fasting blood glucose levels by 26.8 and 20.5%, respectively (Figure 4A). Additionally, we measured plasma biomarkers in MT or DM treated mice. The MT or DM administrated groups showed a significant decrease in total cholesterol compared to the HFD group by 25.1% or 23.4%, respectively (Figure 4B). The levels of plasma free fatty acid in MT and DM treated mice were significantly reduced by 20.6 and 17.4%, respectively (Figure 4C). In addition, DM treatment resulted in a marked reduction of ALT and AST levels (Figure 4D and E), indicating the protecting effects of DM in HFD-induced hepatotoxicity. These results showed that some active compounds from DM might be associated with the improvement of glucose and lipid impairment and effective in preventing hepatotoxicity in the HFD mice.

DISCUSSION

Obesity has emerged as a worldwide health problem and is, therefore, a major focus for therapeutic treatment options. The members of PPAR subfamily have emerged as key pharmacological targets, whose activation can improve metabolic dysfunctions and reduce some cardiovascular risk factors (Guri et al., 2006; Ronnett et al., 2006). Especially, PAPRα was found to play a role in regulating mitochondrial and peroxisomal fatty acid oxidation (Desvergne and Wahli, 1999). Accordingly, PPARα agonist is predicted to be an excellent and novel therapeutic target for obesity and dyslipidemia. In this study, we showed that anti-obesity and hypolipidemic effect of DM is through PPARα transactivation not PPARγ or PPARδ. To confirm which fraction is effective on

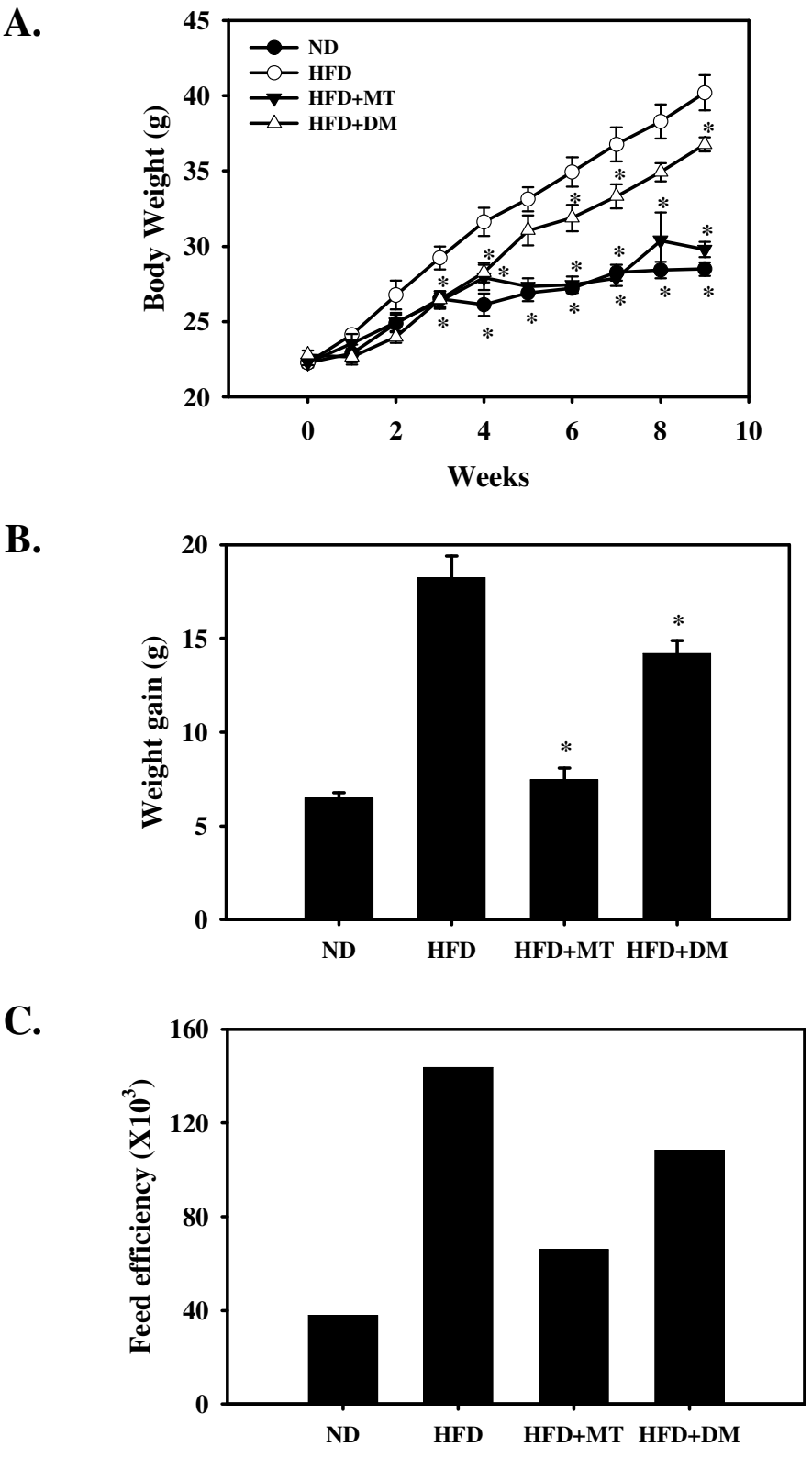

Figure 3. DM treatment significantly reduces body weight gain. Metformin (MT; 500 mg/kg) and Dendrobium moniliforme (DM; 200 mg/kg) were administered to HFD-fed C57BL/6 mice for 9 weeks (n=7). Body weight (A) was measured daily. At the end of the experimental period, weight gain (B) and Feed efficiency (weight gain/food intake) (C) were calculated. Each bar represents the mean ± SD (n=7). *P<0.05 vs. HFD group.

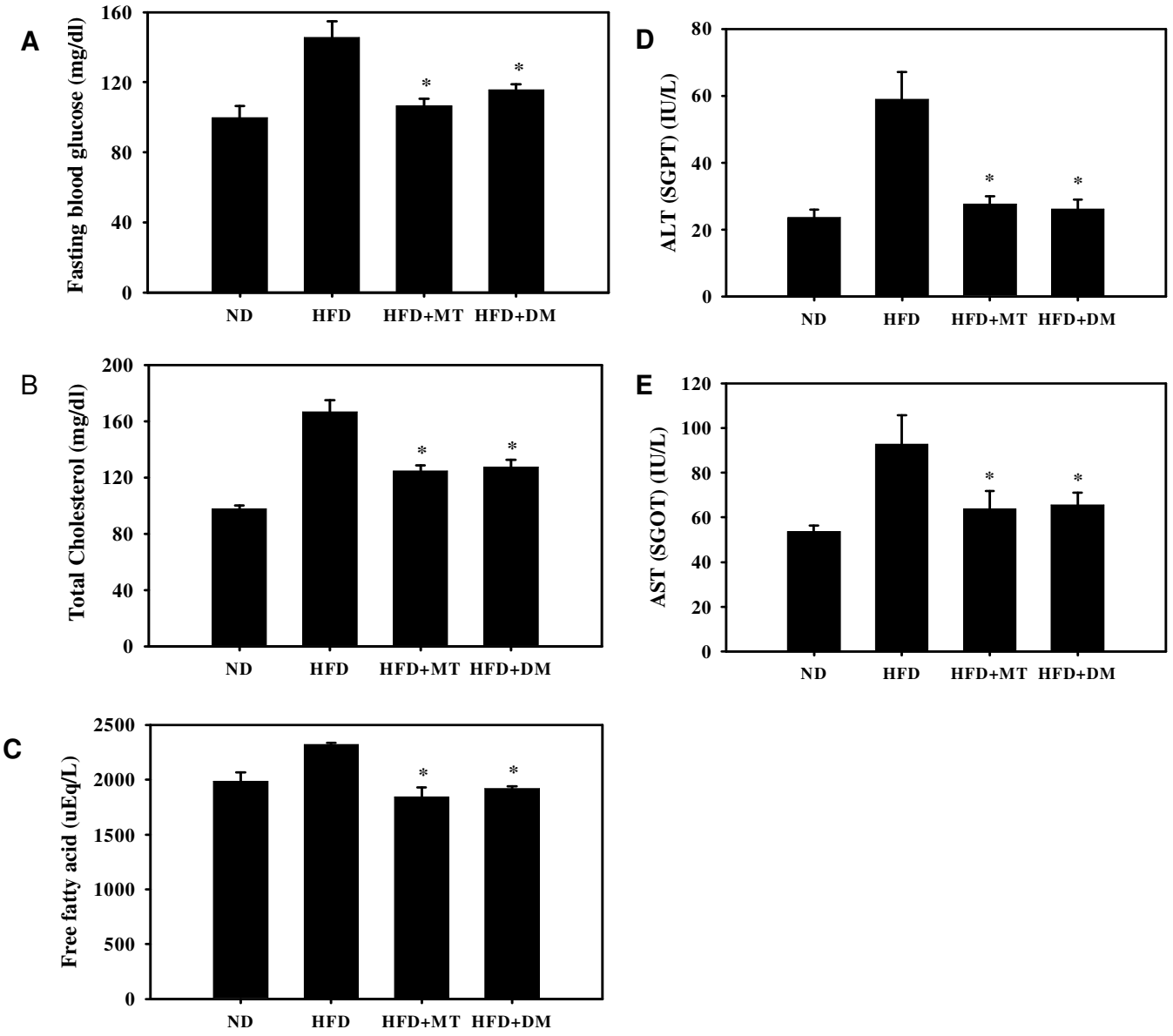

Figure 4. DM improves metabolic markers in blood. Metformin (MT; 500 mg/kg) and *Dendrobium moniliforme* (DM; 200 mg/kg) were administered to HFD-fed C57BL/6 mice for 9 weeks. Fasting blood glucose levels (A) were measured in blood taken from tail vein after 9 weeks of administration. At the end of the experimental period, plasma sample were analyzed for total-cholesterol (B), free fatty acid (C), ALT (D), and AST (E). Each bar represents the mean ± SD (n=7). *P<0.05 vs. HFD

PPARα transactivation, we examined the effects of the five fractions (MC, Hx, EA, BuOH, and DW) from DM extract on transactivation of PPARs, and then identified lipophilic (MC, Hx and EA) fractions as active fractions for PPARα transactivation, implying that some active compounds that activate PPARα may be in the lipophilic fractions. Lipid metabolism regulating effects of DM are confirmed by increased mRNA expression levels of genes involved in fatty acid oxidation such as CPT1, MCAD, ACO2, and ECH1 in human HepG2 hepatocytes. EA fraction from DM significantly increased PPARα transcriptional activity and increased mRNA levels of ACO2 and ECH1 involved in peroxisomal fatty acid β-oxidation in HepG2 cells. This means that some lipophilic components in EA fraction may increase fatty acid β-oxidation in peroxisome through activation of PPARα.

To prove the glucose and lipid regulatory activities of DM, HFD-induced obese mice model was used for further investigation. In this study, MT was used as a positive control because it is the first-line drug of choice for the treatment of type 2 diabetes, in particular, in overweight and obese people. DM (200 mg/kg) administered mice

group for 9 weeks showed significant decrease of body weight gain and feed efficiency compared to the HFD group. Although body weight lowering effect of DM treated group is less effective than that of MT, the fasting blood glucose, total cholesterol, and free fatty acid levels were significantly decreased in DM administered group. In addition, DM treatment significantly reduced serum levels of both ALT and AST to normal level (Figure 4D and 4E) as well as HFD-induced hepatomegaly (data not shown).

In summary, we demonstrate here that DM has beneficial effects on glucose and lipid metabolism in the improvement metabolic disorders by selectively activating PPARα without the severe adverse effects that have been observed in other PPAR agonists, such as hepatotoxicity. Therefore, we strongly suggest that DM would be further developed as therapeutic agents to improve glucose and lipid abnormalities and obesity.

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