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Low molecular weight fucoidan (LMWF) improves ER stress-reduced insulin sensitivity through AMPK activation in L6 myotubes and restores lipid homeostasis in a mouse model of type 2 diabetes

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Running title: LMWF improves ER stress-induced insulin resistance

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Abbreviations: AMPK, AMP-activated protein kinase; ER, endoplasmic reticulum; IRS-1, insulin receptor substrate-1; JNK, c-Jun NH_2-terminal kinase; LMWF, low molecular weight fucoidan; mTOR, mammalian target of rapamycin; PERK, double stranded RNA-activated protein kinase-like ER-associated kinase; PI3K, phosphatidylinositol 3-kinase; S6K1, p70S61 kinase; siRNA, short interference RNA.

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Abstract

Low molecular weight fucoidan (LMWF) is widely used to treat metabolic disorders, but its physiologic effects have not been well determined. In the present study, we investigated the metabolic effects of LMWF in *db/db* mice and the underlying molecular mechanisms involved in endoplasmic reticulum (ER) stress-responsive L6 myotubes. The effect of LMWF-mediated AMP-activated protein kinase (AMPK) activation on insulin resistance via regulation of the ER stress-dependent pathway was examined *in vitro* and *in vivo*. In *db/db* mice, LMWF markedly reduced serum glucose, triglyceride, cholesterol, and LDL levels, and gradually reduced body weights by reducing lipid parameters. Furthermore, it effectively ameliorated glucose homeostasis by elevating glucose tolerance. In addition, the phosphorylation level of AMPK and Akt were markedly reduced by ER stressor, and subsequently, glucose uptake and fatty acid oxidation were also reduced. However, these adverse effects of ER stress were significantly ameliorated by LMWF. Finally, in L6 myotubes, LMWF markedly reduced the ER stress-induced upregulation of mammalian target of rapamycin (mTOR)-p70S61 kinase (S6K) network and subsequently improved the action of insulin via AMPK stimulation. Our findings suggest that AMPK activation by LMWF could prevent metabolic diseases by controlling the ER stress-dependent pathway and that this beneficial effect of LMWF provides a potential therapeutic strategy for ameliorating ER stress-mediated metabolic dysfunctions.

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INTRODUCTION

Fucoidan is a sulfated polysaccharide derived from marine brown algae *Undaria pinnatifida* (Berteau and Mulloy, 2003), and has been demonstrated to regulate glucose homeostasis and hepatic fibrosis in rodent models (Kim et al., 2012; Hayashi et al., 2008). Moreover, recent studies have shown that low molecular weight fucoidan (LMWF) reduces lipid accumulation by retarding the adipogenesis in 3T3-L1 cells (Park et al., 2011). However, although fucoidan is known to regulate many physiological functions (Cumashi et al., 2007), and its effects on insulin resistance and type 2 diabetes are not well understood.

Endoplasmic reticulum (ER) stress has been shown to contribute to obesity, insulin resistance, and type 2 diabetes (Ozcan et al., 2004). Furthermore, studies conducted in the past decade have demonstrated that genetic and dietary obesity are associated with ER stress (Ozcan et al., 2004; Wellen and Hotamisligil, 2005) occurring secondary to increased mTOR activity and increased protein synthesis (Ozcan et al., 2008). The conserved serine/threonine kinase mTOR (mammalian target of rapamycin) integrates inputs from several upstream pathways, including nutritional stimuli, and cellular growth machinery (Kapahi et al., 2010). In a previous report issued by our group, it was shown ER stress-mediated mTOR activation is associated with reduced insulin signaling in muscle cells (Hwang et al., 2012), and a direct link between mTOR/S6K activation and repressed insulin action was suggested to be caused

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by the reversal of this effect by rapamycin (a mTOR inhibitor) in L6 myotubes (Hwang et al., 2012). These observations demonstrate ER stress plays a significant role in the mTOR/S6K-mediated negative-feedback inhibition of insulin activity.

AMP-activated protein kinase (AMPK) is a phylogenetically conserved intracellular energy sensor that has been implicated in the regulation of food intake, body weight, glucose uptake, and lipid metabolism (Carling, 2004; Hardie et al., 2003). AMPK phosphorylates its downstream substrates and thus reduces ATP-consuming anabolic pathways, such as, those responsible for the syntheses of fatty acids, cholesterol, and triacylglycerols, and increases ATP-generating catabolic pathways, such as, fatty acid oxidation and lipolysis (Iglesias et al., 2004; Hardie et al., 2003). Recent reports have suggested that AMPK activation accounts for at least some of the beneficial effects of exercise, such as, increased fatty acid oxidation and glucose uptake (Aschenbach et al., 2004; Merrill et al., 1997; Mu et al., 2001). Furthermore, it has been demonstrated that the activation of AMPK suppresses oxidized LDL-induced ER stress by inhibiting NAD(P)H oxidase-derived ROS production and SERCA oxidation (Dong et al., 2010). Based on these findings, we considered the possibility of a link between the mTOR pathway and ER stress in the contexts of insulin signaling and AMPK activity.

In this study, we examined LMWF to search for a natural AMPK activator and found that LMWF activated AMPK. When the antidiabetes effects of LMWF were explored in obese diabetic mice (*db/db* mice), LMWF ameliorated hyperlipidemia and insulin resistance

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in vivo. Strikingly, LMWF reduced endoplasmic reticulum (ER) stress in the muscle tissue of *db/db* mice as well as in tunicamycin-incubated L6 myotubes and improved insulin signaling. Base on these results, it appears that LMWF activates AMPK, and alleviates ER stress, making it a potentially new treatment for metabolic dysfunctions.

MATERIALS AND METHODS

Materials

Tunicamycin (Hwang et al., 2012), 2-deoxy-glucose, compound C (Zhou et al., 2001), wortmannin, and rapamycin (Hwang et al., 2012) were purchased from Calbiochem (Darmstadt, Germany). Metformin (Bailey and Turner, 1996), insulin, BSA, and chemicals were purchased from Sigma (St. Louis, MO, USA). All other materials were purchased from the companies indicated.

Polysaccharides

LMWF was donated by Bion Co. Ltd (Daegu, Korea) and was obtained by the acid hydrolysis of high molecular weight fucoidan (HMWF) extracts from brown seaweed, *Undaria pinnatifida*, as previously described (Park et al., 2010).

Animal experimental procedures

Male C57BL/KsJ-*Lepr*^{db}/*Lepr*^{db} (*db/db*) mice and lean C57BL/6J*ms* (WT or Lean) mice purchased from Jackson Laboratories were housed in a temperature-controlled room (22 ± 2

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^{o}C) under a 12 h light/dark cycle. From 8 weeks of age, two different dosages of LMWF (250 or 500 mg/kg) were orally administered daily for 6 weeks to *db/db* mice. After treatment with LMWF, changes in body weight, food intake, epididymal fat weight, blood glucose levels, serum leptin and adiponectin contents, fat adiponectin contents, serum HDL, LDL, triglyceride and total cholesterol levels were measured in blood samples or tissues as appropriate. All animal studies and the study protocol were approved by the Institutional Animal Use and Care Committee (IAUCC) of Yeungnam University.

Metabolic parameters

Plasma glucose was measured using a glucometer (Roche Diagnostics, Mannheim, Germany) in blood collected from a tail vein, as previously described (Um et al., 2004). For glucose tolerance testing, a single dose of 2.5 μg/kg glucose was injected intraperitoneally after a 14 h fast. Blood glucose was measured at the indicated times. All blood samples were collected from mice fasted for 12 h and plasma samples were immediately prepared. Triglycerides, total cholesterol, LDL, and HDL levels were measured using an automated blood analyzer (AU400, Olympus, Japan), and adiponectin and leptin levels were detected using a commercially available ELISA kit (Otsuka Pharm., Japan) or a radioimmunoassay kit (Linco Research, USA), respectively, as described previously (Fujita et al., 2005; Sahai et al., 2004).

Histological analysis

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Histological analysis was performed as described previously (Hong et al., 2004; Villena et al., 2004). Briefly, 6 weeks after administering LMWF (250, 500 mg/kg) and/or metformin (the positive control; 250 mg/kg), mice were sacrificed. Liver tissues were collected by perfusion, fixed in 4% paraformaldehyde, and embedded in paraffin. Sectioned at 4 μm, and stained with Oil-red O. Cell size was measured following DakoCytomation (Dako co, CA, USA), according to the manufacturer's instructions.

Cell culture

L6 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were maintained in α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Cells were allowed to differentiate into myotubes as reported previously (Hwang et al., 2008).

Glucose uptake assay

Radiolabeled 2-deoxyglucose uptake was assayed as described previously (Hwang et al., 2008).

Determination of triglyceride and cholesterol contents

Triglycerides and total cholesterol contents in cell lysates were determined using a colorimetric assay (Wang et al., 2003); results (μg of lipid/mg of cellular protein) are expressed as percentages of non-treated controls. In brief, L6 myotubes were maintained in

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serum-free medium overnight and incubated for 16 h in the absence or presence of 150 μg/ml of LMWF. Cell lysates were prepared as described above. Triglycerides and total cholesterol levels in cell lysates were measured using Infinity^{TM} reagents (Thermo DMA, Louisville, CO. USA), according to the manufacturer's instructions.

Fatty acid oxidation

Palmitate oxidation was analyzed as described previously (Cabrero et al., 2001).

Immunoblotting

L6 myotubes and tissues samples were isolated and processed as described previously (Hwang et al., 2008). Membranes were probed with LKB1, phospho-AMPK (pAMPK), AMPK, pACC, ACC, pPERK, PERK, pIRE-1, IRE-1, pJNK, JNK, pmTOR, mTOR, pS6K, S6K, p4E-BP, 4E-BP, pAkt, Akt (all from Cell Signaling Technology, Beverly, MA, USA), and β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then developed using an ECL Western blot detection kit (Amersham Bioscience, Piscataway, NJ, USA).

Transfection with small-interfering RNA (siRNA)

For siRNA experiments, SMARTpool for rat LKB1 (L-100539-01-0020), rat AMPKα2 (L-100623-00-0020) was obtained from Dharmacon (Lafayette, CO, USA). Non-specific siRNA (ONTARGET*plus* siCONTROL Non-Targeting Pool, D-001810-10-20) was used as a control. For transient expression experiments, L6 myotubes were serum starved for 16 h in serum-free media, and then transfected with DharmaFECT transfection reagent (Dharmacon)

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in 12-mm or 60-mm plates containing 100 nmol/l of either LKB1 siRNA (si LKB1), AMPKα2 siRNA (siAMPK) or non-targeting control siRNA (Mock) per plate according to the manufacturer's protocol. After 48 h, cells were treated with vehicle or LMWF for 2 h, then with tunicamycin (5 μg/ml) for 3 h, and finally, with insulin (100 nmol/l) for 10 min. Cells were then subjected to immunoblot or glucose uptake experiments.

Statistical analysis

Data are expressed as means ±SEMs. Analysis of variance (ANOVA) and/or the Student's *t* tests were used to determine the significances of differences. *P* values of < 0.05 were considered statistically significant.

RESULTS

Effects of LMWF on dyslipidemia in *db/db* mice

To examine the *in vivo* metabolic effects of LMWF on diabetes, C57BL/KsJ-*db/db* (*db/db*) mice were treated orally for 6 weeks with LMWF (250, 500 mg/kg). Metformin (an AMPK activator; 250 mg/kg) was used as a positive control in animal experiments. The body weights of LMWF-treated animals were slightly lower than those of metformin and untreated controls, but food intake in these three groups were not significantly different (Fig. 1A, B). As shown in Fig. 1C, gross observations of LMWF-treated mice showed a significantly less white adipose tissue and more small adipocytes in epididymis fat pad than in untreated *db/db*

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mice. Furthermore, of the abdominal adipocytes of untreated *db/db* mice exhibited severe hypertrophy as compared with those of wild type controls. In addition, adipocyte diameters were markedly lower in LMWF-treated mice and in metformin-treated mice than in untreated *db/db* controls (Fig. 1D). We next examined the effects of LMWF on lipid metabolic parameters in *db/db* mice. Serum cholesterol and triglyceride levels were lower in LMWF-treated than in untreated *db/db* mice (Fig. 1E, F), as were serum LDL cholesterol levels (Fig. 1G). However, neither LMWF nor metformin affected serum HDL cholesterol levels (Fig. 1H). Oil Red O staining showed that fatty droplet sizes were markedly lowered by LMWF and by metformin (Fig. 1I). These results suggest that LMWF reduces fat mass primarily by reducing the sizes of fat cells rather than fat cell number. Taken together, these results demonstrate that LMWF controls lipid metabolic profiles in *db/db* mice.

LMWF reduced glucose homeostasis and serum adipokine levels via AMPK activation in *db/db* mice

To determine the physiological role of LMWF on the regulations of glucose metabolism and adipokine levels, LMWF (250, 500 mg/kg) or metformin (250 mg/kg) were administered once to *db/db* mice. As shown in Fig. 2A and B, LMWF gradually reduced blood glucose levels and significantly improved glucose tolerance as compared with untreated *db/db* controls, and LMWF or metformin markedly reduced insulin levels (Fig. 2C). Furthermore, serum leptin levels fell further in LMWF-treated diabetic mice than in untreated *db/db*

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controls (Fig. 2D). Serum adiponectin levels are closely related to systemic insulin sensitivity, and a depressed serum adiponectin level is considered a feature of obesity and type 2 diabetes (Cnop et al., 2003; Weyer et al., 2001). As was expected, serum adiponectin levels were significantly higher in LMWF and in metformin *db/db* mice than in untreated controls (Fig. 2E). Similarly, adiponectin levels in adipose tissue were also higher LMWF and metformin *db/db* mice than in untreated controls (Fig. 2F). Next, we confirmed whether the AMPK/mTOR/S6K/JNK pathway (a crucial regulator of metabolic dysfunction) are affected by LMWF in diabetic mice. As was expected, LMWF significantly elevated the AMPK-ACC axis as compared with untreated controls, and subsequently dose-dependently depressed the mTOR, S6K, 4E-BP, PERK, IRE-1, and JNK pathways (Fig. 2G), which is consistent with the observed elevations of glucose homeostasis and lipid parameters by LMWF. Overall, these results suggest that LMWF improves glucose metabolism and adipokine profiles in *db/db* mice.

LMWF improved glucose and lipid metabolism via AMPK activation in L6 myotubes

The activation of AMPK involves the phosphorylation of Thr172 in the activation domain of its catalytic α subunit (Hardie et al., 1998; Hayashi et al., 2000). Accordingly, we investigated whether LMWF affects the phosphorylation of AMPK at this locus. It was found that, like metformin, LMWF stimulated AMPK activation in differentiated L6 myotubes. Moreover, LMWF significantly and time-dependently increased the phosphorylations of

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AMPK (Thr172) and ACC (Ser79) (Fig. 3A), and effectively dose-dependently stimulated the AMPK-ACC axis in L6 myotubes (Fig. 3B). We next examined the effect of LMWF on glucose uptake in L6 myotubes, because one of the major acute effects of AMPK is to stimulate muscle glucose uptake (Bergeron et al., 1999). Glucose uptake was increased by LMWF, in a manner similar to that observed for metformin (Fig. 3C). LMWF or metformin markedly reduced intracellular triglyceride and cholesterol content (Fig. 3D, E). Recently, the activation of AMPK by metformin was shown to reduce intracellular lipid contents and to increase fatty acid oxidation in liver cells and tissues (Hawley et al., 2002; Zang et al., 2004). Accordingly, we next examined the effect of LMWF on fatty acid oxidation in L6 myotubes using radioisotope-labeled palmitate. As was expected, fatty acid oxidation was enhanced by both LMWF and metformin versus untreated controls (Fig. 3F). Taken together, these findings show that LMWF controls glucose and lipid profiles in muscle cells.

LMWF controlled glucose and lipid contents through AMPK

To investigated the effects of LMWF on AMPK activity, we treated L6 myotubes with compound C (Com C; an inhibitor of AMPK), as reported previously (Zhou et al., 2001). LMWF or metformin significantly elevated glucose uptake in L6 myotubes versus untreated controls, and this was markedly prevented by Com C (Fig. 4A). Furthermore, LMWF or metformin also markedly reduced triglyceride and cholesterol contents in L6 myotubes, and these inhibitory effects were prevented by Com C (Fig. 4B, C). Interestingly, LMWF or

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metformin significantly elevated fatty acid oxidation, and this was markedly reduced by pretreated with Com C (Fig. 4D). Furthermore, siRNA-mediated knockdown of AMPKα2 completely abolished glucose uptake and fatty acid oxidation by LMWF or metformin (Supplemental Fig. 1). LKB1 is the major upstream kinase of AMPK and regulate AMPK activity (Sakamoto et al., 2005). To determine which upstream kinase, LKB1, is primarily involved in the activation of AMPK by LMWF, we treated L6 myotubes with LKB1-specific siRNA. Interestingly, this silencing of LKB1 reduced the phosphorylation of AMPK and ACC by LMWF (Supplemental Fig. 2A). Furthermore, LMWF-stimulated glucose uptake was also reduced by siRNA LKB1 (Supplemental Fig. 2B).

Overall, activation of the LKB1/AMPK pathway was found to have a positive impact on glucose and lipid homeostasis in L6 myotubes due to improved glucose uptake and fatty acid oxidation.

LMWF improved ER stress-induced insulin resistance in L6 myotubes

Previous studies have shown that tunicamycin promotes the activations of ER stress markers, such as, RNA-activated protein kinase-like ER resident kinase (PERK), inositol-requiring kinase-1 (IRE-1), c-Jun NH_2-terminal kinase (JNK), and mTOR-S6K, and that these activations reduces insulin signaling (Ozcan et al., 2004; Ozcan et al., 2006; Um et al., 2004). Based on these findings, we investigated the relation between ER stress and the action of insulin in L6 myotubes. ER stress-dependent signal pathways, such as, PERK, IRE, JNK,

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and mTOR-S6K were markedly increased by tunicamycin as compared with untreated controls (Fig. 5A). Interestingly, although the insulin-mediated induction of Akt phosphorylation and basal AMPK phosphorylation are dramatically inhibited under ER stress, we found that this effect was abolished by LMWF (Fig. 5A). A previous report showed that insulin plays a central role in glucose transport via the activations of phosphatidylinositol 3-kinase (PI3K) and Akt in muscle cells (Wang et al., 1999). In the present study, insulin-induced glucose uptake was markedly reduced by tunicamycin and significantly increased by LMWF in untreated controls (Fig. 5B). Moreover, improved glucose uptake by LMWF was blocked by wortmannin (a specific PI3K inhibitor) (Fig. 5B). Furthermore, the ER stress-mediated inhibition of fatty acid oxidation was significantly increased by LMWF, and this phenomenon was reversed by Com C pretreatment, confirming the effect of LMWF on fatty acid oxidation in L6 myotubes (Fig. 5C). However, although LMWF decreased the activations of PERK, IRE-1, and of JNK in muscle tissues from *db/db* mice (Fig. 2G), it had no modulatory effect on the activations of PERK, IRE-1, or JNK in tunicamycin-treated L6 myotubes (Fig. 5A). On the other hand, ER stress-induced phosphorylations of mTOR and S6K were dramatically lower by LMWF (Fig. 5A). These findings show that LMWF has an important effect on the ER stress-mediated mTOR-S6K pathways, which suggests that this inhibitory effect is associated with the improved insulin sensitivity observed following LMWF treatment. Similar results were obtained for rapamycin, a potent inhibitor of mTOR

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(Fig. 5B, D), which in turn suggests that the insulin-sensitizing effect of rapamycin is the results of its directly targeting the mTOR-S6K pathway. Overall, these results suggest that LMWF improves ER stress-induced insulin resistance via an AMPK-dependent pathway.

LMWF was essential for the preservation of insulin sensitivity via an AMPK-dependent pathway

To investigate whether the effects of LMWF in ER-stressed L6 myotubes are mediated via an AMPK pathway, we examined the effect of siRNA-mediated knockdown of AMPKα2 in L6 myotubes. As was expected, AMPKα2 siRNA significantly decreased the protein levels and the activity of α2 AMPK (Fig. 6A). Furthermore, AMPK was highly phosphorylated by LMWF, but AMPKα2 siRNA this prevented the phosphorylation where control siRNA did not (Fig. 6A). Moreover, AMPKα2 siRNA completely abolished LMWF preserved insulin-stimulated Akt phosphorylation and glucose uptake under ER stress (Fig. 6A, B). These results suggest that AMPK activation is required for LMWF-mediated increase in Akt phosphorylation and glucose uptake under ER stress. Furthermore, LMWF induced fatty acid oxidation under ER stress, but this was completely blocked by AMPKα2 siRNA pretreatment (Fig. 6C). In addition, the ER stress-mediated phosphorylations of mTOR and S6K were markedly repressed by LMWF, and this repression was prevented by AMPKα2 siRNA (Fig. 6A). Overall, these results suggest that an increase in AMPK activity by LMWF is required to regulate the insulin signaling pathway and glucose uptake under ER stress. Since serine

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phosphorylation of IRS1 by mTOR-S6K has recently shown to interrupt the interaction between IRS1 and PI3K, and thus, to reduce PI3K activity and PI3K-mediated downstream events (Tzatsos and Kandror, 2006), our findings strongly suggest that, under ER stress, the up-regulation of AMPK by LMWF promotes insulin sensitivity by controlling an ER-stress-mediated signaling pathway.

DISCUSSION

LMWF has been previously reported to ameliorate the progression of atherosclerosis in patients with type 2 diabetes mellitus (Doronina et al., 2007). In the present study, we found that LMWF improves glucose homeostasis and lipid profiles in *db/db* mice and ER stress-induced insulin resistance in L6 myotubes in a manner similar to metformin. Furthermore, LMWF was found to acutely activate the LKB1/AMPK pathway, and thus, to stimulate glucose uptake and fatty acid oxidation in muscle cells.

The physiological relevances of the contributions made by LMWF to glucose and lipid profiles were investigated in *db/db* mice, which are hyperleptinemic and subsequently become obese. Furthermore, these mice have been used to test the efficacies of pharmaceuticals against obesity, type 2 diabetes, and insulin resistance (Nakagawa et al., 2003; Neary et al., 2005). In the present study, body weight decreases were observed within 14-42 days in *db/db* mice after initiating LMWF treatment, and lipid parameters, fat weights,

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and serum leptin and triglyceride levels were dramatically decreased (Figs. 1 and 2). Previous studies have demonstrated excessive adipose tissue leads to increased fat accumulation and adipose cell hypertrophy and that it reduces adiponectin levels in obese rodents and humans (Morange et al., 2000; Arita et al., 1999). Furthermore, hypoadiponectinemia is known to be strongly associated with insulin resistance (Mitchell et al., 2005). In the present study, LMWF effectively reduced adipose tissue amounts and adipose cell hypertrophy, and significantly improved the leptin and adiponectin contents in the adipocytes in *db/db* mice (Fig. 2). Hyperglycemia is a major feature of diabetes, and must be controlled when treating diabetes (Sathishsekar and Subramanian, 2005). Our findings show that LMWF benefits glucose homeostasis by reducing blood glucose levels and improving glucose tolerance (Fig. 2). Diabetes generally exacerbates hyperlipidemia in *db/db* mice (Tuman and Doisy, 1977), and thus, increases lipid contents, including serum LDL, triglyceride, and total cholesterol levels, and decreases HDL levels (Forrester et al., 2005). We found that LMWF markedly reduced serum LDL, triglyceride and total cholesterol levels but did not affect serum HDL levels in *db/db* mice (Fig. 1), which suggests LMWF might be effective at ameliorating metabolic abnormalities. However, more detailed investigations are required to study the effects of LMWF on insulin resistance are required in other animal models, such as, in AMPK null mice and models of diet-induced metabolic dysfunction.

ER stress is receiving more attention because it has been linked to the pathogenesis of type

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2 diabetes and to the inhibition of insulin signaling (Ozcan et al., 2004; Ozcan et al., 2006). ER stress also plays a crucial role in the loss of AMPK activity caused by ER stress inducers, such as, thapsigargin and tunicamycin (Rahman et al., 2009). Thus, agents that alleviate AMPK activation may prevent ER stress-induced insulin resistance (Dong et al., 2010). Therefore, we examined the effect of LMWF on AMPK phosphorylation. We found that treatment with LMWF significantly stimulated the AMPK-ACC axis both time- and dose-dependently, and subsequently elevated glucose uptake and fatty acid oxidation in L6 myotubes, and that these effects of LMWF were abolished by pretreatment with compound C, an inhibitor of AMPK (Fig. 3 and 4). Plasma concentrations of LMWF measured using a liquid chromatography coupled to tandem mass spectrometry at steady state range 18~30 μg/ml and 38~46 μg/ml at the dose of 250 and 500 mg/kg, respectively, in mice (data not shown). The concentrations (150 μg/ml) of LMWF exerting the maximum pharmacodynamic effects in L6 myotubes were comparable to the area under the plasma LMWF concentration-time curve at steady state (AUC_{τ}, ~576 μg·h/ml at the doses of 250 mg/kg) which represents the systemic exposure of the compound in mice following a 6-week repeated oral administration once daily.

As mentioned above, tunicamycin was found to stimulate ER stress markers, PERK, IRE-1, JNK, mTOR, and S6K and to repress insulin-stimulated Akt activation and glucose uptake (Fig. 5A, B). On the other hand, LMWF enhanced the action of insulin and reduced

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the ER stress-mediated mTOR-S6K axis, which we found to be correlated with S6K activity (Fig. 5A). Because activation of the mTOR-S6K axis has been shown to down-regulate insulin-stimulated Akt phosphorylation and glucose uptake (Tzatsos and Kandror, 2006), these results suggest that LMWF improves insulin sensitivity by down-regulating the mTOR-S6K axis. Interestingly, rapamycin-mediated increases of Akt phosphorylation and glucose uptake were found to be correlated with reduced S6K phosphorylation, which in turn, was found to be correlated with S6K activity under ER stress (Fig. 5B, D). These results suggest that rapamycin (a mTOR pathway inhibitor) acts as a key regulator of insulin sensitivity by down-regulating the S6K-dependent pathway, which is consistent with the beneficial effects of LMWF in muscle cells. In skeletal muscle, AMPK directly phosphorylates at least two proteins and rapidly suppresses the activities of mTORC1 (a TSC2 tumor suppressor) and the critical mTORC1 binding subunit Raptor. Furthermore, activated AMPK has been suggested to inhibit mTORC1-dependent phosphorylations of S6K1 and 4E-BP1 (Inoki et al., 2003). Our findings demonstrated that the AMPK downregulation of mTORC1 could help explain the insulin-sensitizing effects of AMPK activators. Because mTORC1 activity dictates the extent of feedback inhibition of PI3-kinase activity, AMPK activation actually serves to attenuate this feedback and to promotes autonomous restoration of IRS protein levels in cells and IRS signaling to PI3-kinase. The net effect is most clearly observed on IRS protein levels and Akt activation (Harrington et al., 2004; Shah et al., 2004). When mTORC1 activity is

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high, IRS protein levels are low and Akt is inhibited, but when AMPK is activated, it suppresses mTORC1, and thus, restores IRS protein levels and Akt activation.

AMPK (Hayashi et al., 2000) and insulin receptor signaling pathways (Krook et al., 2004; Saltiel and Kahn, 2001) importantly regulate glucose homeostasis in muscle cells. Activated insulin receptor transduces insulin action by stimulating PI3K-Akt pathway, and subsequently promotes glucose uptake in muscle cells. In a previous study, we attempted to ameliorate insulin resistance by controlling the up-regulation of the PI3K-Akt axis and restoring insulin sensitivity (Chang et al., 2004). In the present study, glucose uptake results indicated that LMWF or rapamycin might be able to restore the ER stress-mediated inhibition of insulin sensitivity by promoting insulin signaling, glucose transport, and fatty acid oxidation (Fig. 5). Although it is possible that ER stress adversely affects the AMPK-ACC pathway in L6 myotubes, our findings show that LMWF enhances insulin sensitivity by stimulating an AMPK-dependent pathway, which concurs with previous reports (Iglesias et al., 2004; Merrill et al., 1997). Furthermore, we found that by controlling this AMPK-dependent pathway in L6 myotubes, LMWF reduced intracellular triglyceride and cholesterol levels (Fig. 3 and 4). Thus, our findings suggest that LMWF reduces lipid levels in L6 myotubes, which is consistent with the stimulation of the AMPK-ACC axis by LMWF, and implies a link between lipolysis and lipogenesis events. However, additional studies are required to determine to what extent the lipid-lowering effects of LMWF are due to; 1) the

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phosphorylation of AMPK in AMPK knockout mice, and 2) to effects of insulin signaling via AMPK on the mTORC1-S6K axis.

In conclusion, our results suggest LMWF reduces whole-body adiposity and improves lipid and glucose homeostasis, and imply that LMWF improves ER stress-induced insulin resistance, at least in part, by activating AMPK both *in vitro* and *in vivo*. This mechanism of insulin resistance improvement via the LMWF-AMPK pathway provides new insights of means of alleviating the effects of ER stress-mediated metabolic dysfunction, and hopefully, aid the development of novel therapeutic agents for the treatment of obesity, insulin resistance, and type 2 diabetes.

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Authorship Contributions

Participated in research design: Hwang, Y.D. Kim, and Jeong.

Conducted experiments: Jung, Park, D.S. Lee, Ku, Li, Lu, Chao, K.J. Kim, J.Y. Lee, Baek, and Kang.

Contributed new reagents or analytic tools: Baek, Kang, and Ku.

Performed data analysis: Hwang, Y.D. Kim, and Chang.

Wrote or contributed to the writing of the Manuscript: Hwang, Y.D. Kim, and Chang.

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FOOTNOTES

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Seung-Lark Hwang and Hyeun Wook Chang contribute equally to the design and preparation of the manuscript.

Yong-Tae Jeong and Yong Deuk Kim contribute equally to this work.

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LEGENDS FOR FIGURES

Figure 1. Effect of LMWF on body weight and lipid profiles in *db/db* mice. Mice were treated for 6 weeks with different doses of LMWF (250, 500 mg/kg) or metformin (250 mg/kg). Effects of LMWF on body weight (A) and food intake (B). Effect of LMWF on weight of white adipose tissue. Histological analysis was performed by hematoxylin and eosin staining (C). Effect of LMWF on the morphology of white adipose tissue deposits (thicknesses of deposited fat pads). Histological analysis was performed by hematoxylin and eosin staining. Arrows indicate diameters of adipocytes. All H&E stained; Scale bars = 80 μm (D). Effects of LMWF on serum lipid parameters (E-H). Effect of LMWF on fatty droplets in liver tissues. Histological analysis was performed by Oil Red O staining (I). Metformin (Met, 250 mg/kg) was used as a positive control. Mice were allocated to experimental groups (n=5-7 mice per group). Wild-type (Lean, C57BL/6J*ms*, filled circle); *db/db* (*db*, unfilled circle), *db/db* + metformin (*db/db* + M, unfilled triangle), *db/db* + LMWF 250 (*db/db* + F2, unfilled square), *db/db* + LMWF 500 (*db/db* + F5, filled square). *****$P < 0.05$ *vs*. untreated *db/db* mice.

Figure 2. LMWF improved glucose metabolism and adipokine profiles in *db/db* mice. Mice were treated for 6 weeks with LMWF (250, 500 mg/kg) or metformin (250

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mg/kg). Effect of LMWF on fasting blood glucose levels (A) and intraperitoneal glucose tolerance test results (B) after glucose injection. Effect of LMWF on serum insulin (C), leptin (D), adiponectin levels (E). Effect of LMWF on adiponectin levels in adipose tissues (F). Under same conditions, whole cell extracts were isolated from skeletal muscle and analyzed by Western blotting with various antibodies, and then normalized versus non-phosphorylated forms (total forms) (G). Metformin (Met, 250 mg/kg) was used as a positive control. Experimental groups were composed of 5 to 7 mice. *****$P < 0.05$ *vs*. untreated *db/db* mice.

Figure 3. LMWF stimulated glucose uptake and fatty acid oxidation via AMPK phosphorylation in L6 myotubes. (A) L6 myotubes were stimulated with LMWF (150 μg/ml) for the indicated times. (B) L6 myotubes were incubated in serum-free medium overnight and stimulated for 2 h with various concentrations of LMWF. Under these conditions, whole cell extracts (40 μg/lane) were isolated and analyzed by immunoblotting using the indicated antibodies, and then normalized to versus β-tubulin and/or non-phosphorylated forms (total forms). (C) L6 myotubes were serum-deprived and then incubated with the indicated concentrations of LMWF and metformin for 2 h, respectively. Glucose uptakes were measured. Levels of intracellular triglyceride (D) and cholesterol (E) in cells treated with LMWF for 24 h were determined by

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spectrophotometrically and results are expressed as μg of lipid/mg of protein. (F) L6 myotubes were incubated for 24 h with either vehicle or 150 μg/ml of LMWF. Fatty acid (9, 10-[^{3}H]palmitate) oxidation was determined by measuring counts per minute of ^3H_2O produced per milligram of protein per hour. Metformin (Met, 2 mmol/l) was used as a positive control. All results are representative of at least three independent experiments. ***P* < 0.05 *vs*. untreated controls.

Figure 4. LMWF improved glucose uptake and lipid contents in a AMPK-dependent manner. (A) For glucose uptake experiments, L6 myotubes were treated with LMWF or metformin for 2 h and then treated with compound C (10 μmol/l, Com C) for 20 min. Levels of intracellular triglyceride (B) and cholesterol (C) and fatty acid oxidation (D) are shown. All results are representative of at least three independent experiments. ***P* < 0.05, ****P* < 0.01 versus untreated controls.

Figure 5. LMWF prevented insulin action and fatty acid oxidation under ER stress. (A) L6 myotubes were pretreated with LMWF for 2 h and tunicamycin (Tuni, 5 μg/ml) for 3 h and then stimulated for 10 min with insulin (100 nmol/l, Ins). Whole cell extracts were isolated and analyzed by Western blotting using various antibodies. Protein production was normalized versus non-phosphorylated forms (total forms). (B) Cells

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were pretreated for 30 min with wortmanin (Wort, 100 nmol/l) and treated LMWF for 2 h or rapamycin (Rapa, 100 nmol/l) for 20 min and tunicamycin (Tuni, 5 μg/ml) for 3 h and then stimulated for 10 min with insulin (100 nmol/l, Ins). Glucose uptakes were determine described in Methods. All results are representative of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, and $^{\&}P < 0.05$ versus untreated controls, Ins-treated cells, Ins- and Tuni-treated cells, Ins-, Tuni- and LMWF-treated cells. (C) As described above, fatty acid oxidation under ER-stress was measured as counts per minute of $^{3}H_2O$ produced per milligram of protein per hour. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$ versus untreated controls, Tuni-treated cells, or Tuni- and LMWF-treated cells. (D) L6 myotubes were pretreated with rapamycin and treated with tunicamycin and then treated with insulin. Whole cell extracts were isolated and analyzed by immunoblotting using the indicated antibodies, and then normalized with respect to non-phosphorylated forms (total forms).

Figure 6. Stimulation of glucose uptake and fatty acid oxidation by LMWF was mediated by AMPK. (A) L6 myotubes were infected with control siRNA (mock) or AMPKα2 siRNA (si AMPK) for 48 h. Cells were then pretreated with LMWF and treated with tunicamycin, and then treated with insulin. Whole cell extracts were isolated and analyzed by Western blotting using various antibodies. Protein production

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was normalized with respect to non-phosphorylated forms (total forms). Under the same conditions, glucose uptake (B) and fatty acid oxidation (C) were measured as described in Methods. All results are representative of at least three independent experiments. ***P < 0.05, ****P < 0.01, $^{\#}P$ < 0.05 versus untreated controls, Ins-treated cells, Tuni-treated cells, Ins- and Tuni-treated cells, or Tuni- and LMWF-treated cells.

Figure 1

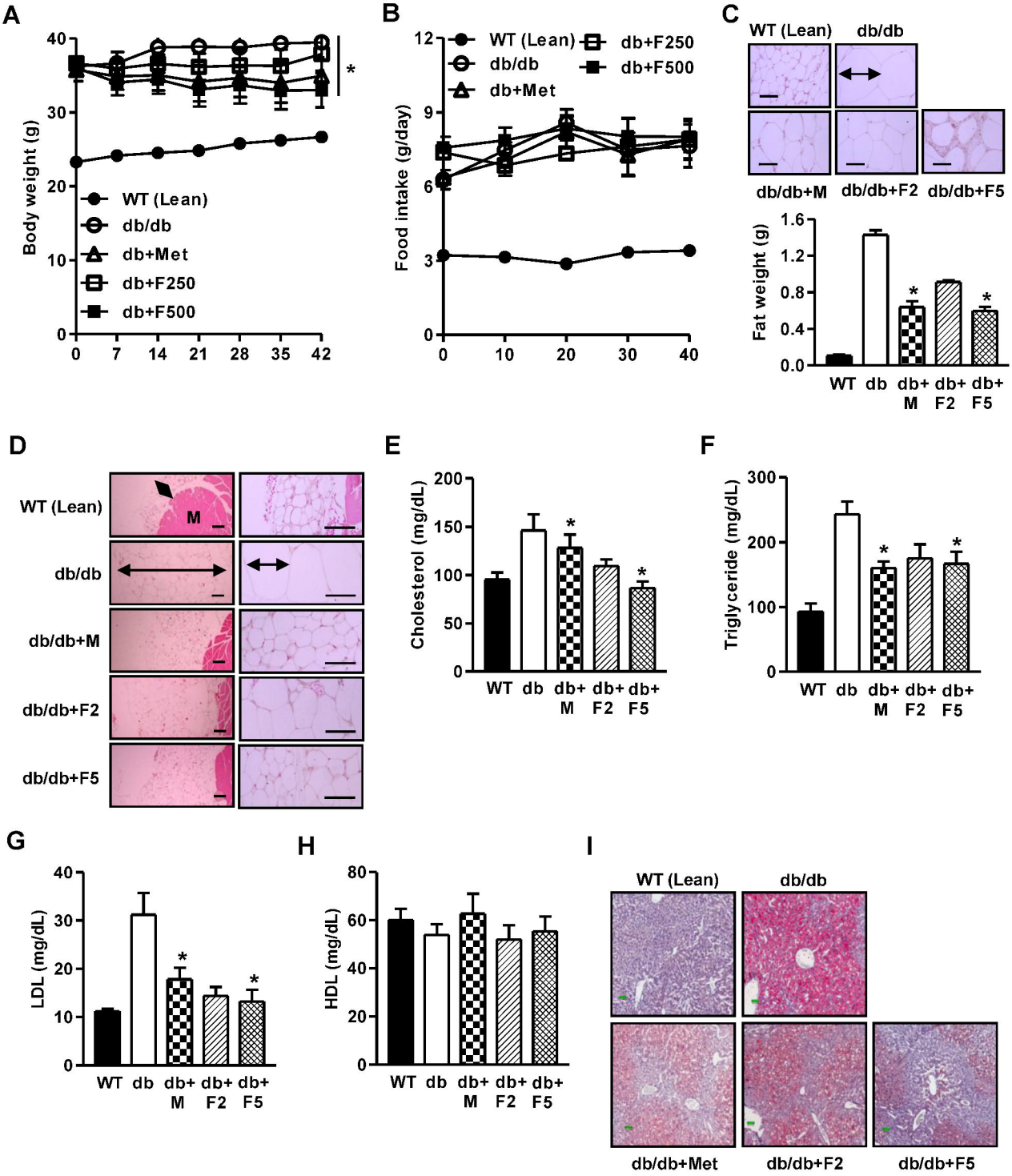

A
Body weight (g)
WT (Lean)
db/db
db+Met
db+F250
db+F500
*
B
Food intake (g/day)
WT (Lean)
db/db
db+Met
db+F250
db+F500
C
WT (Lean)
db/db
db/db+M
db/db+F2
db/db+F5
Fat weight (g)
WT
db
db+ M
db+ F2
db+ F5
D
WT (Lean)
db/db
db/db+M
db/db+F2
db/db+F5
M
E
Cholesterol (mg/dL)
F
Triglyceride (mg/dL)
G
LDL (mg/dL)
H
HDL (mg/dL)
I
WT (Lean)
db/db
db/db+Met
db/db+F2
db/db+F5

Figure 2

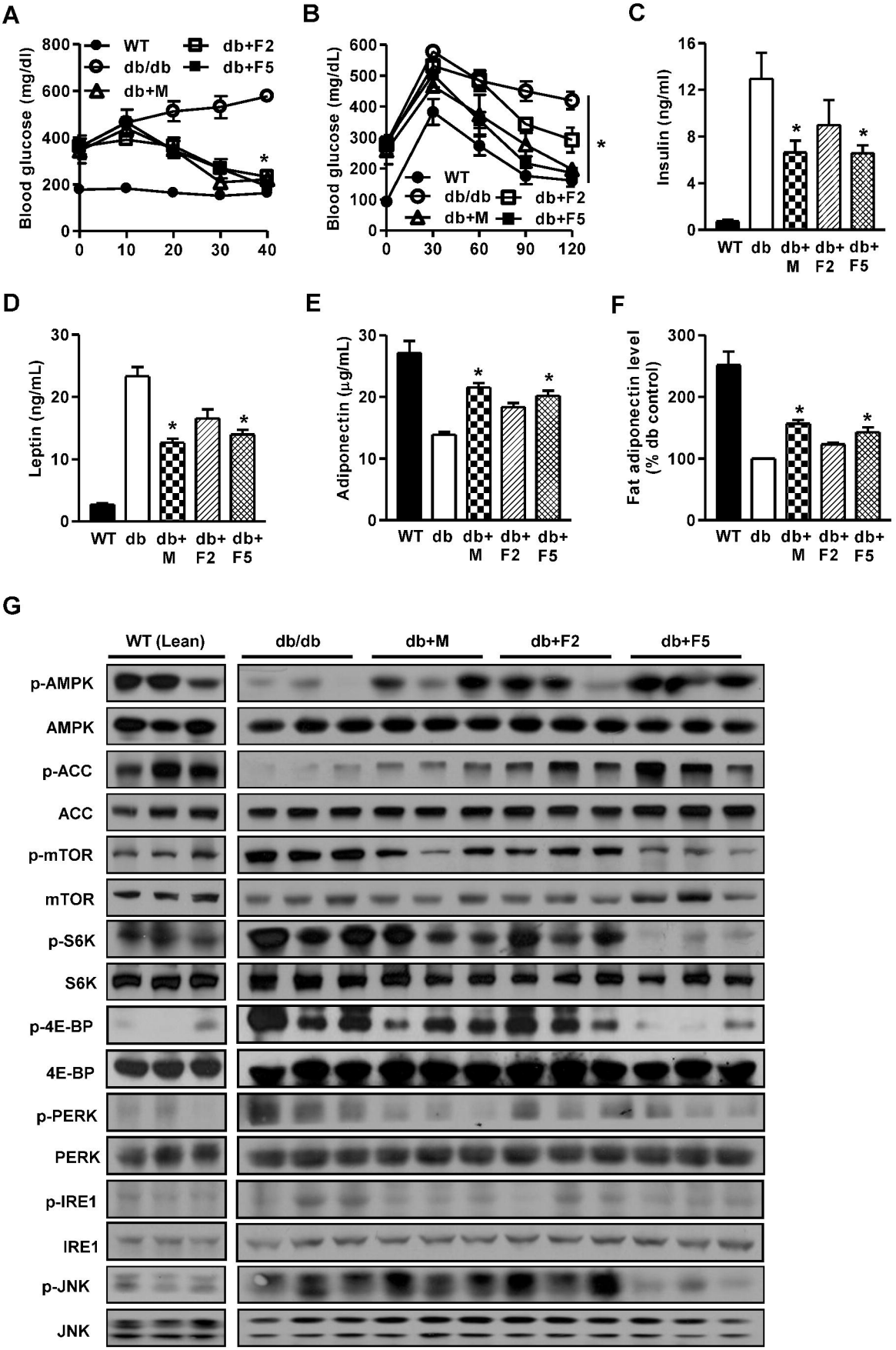

A
Blood glucose (mg/dl)
WT
db/db
db+M
db+F2
db+F5
B
Blood glucose (mg/dL)
C
Insulin (ng/ml)
D
Leptin (ng/mL)
E
Adiponectin (μg/mL)
F
Fat adiponectin level (% db control)
WT db db+ M db+ F2 db+ F5
G
WT (Lean)
db/db
db+M
db+F2
db+F5
p-AMPK
AMPK
p-ACC
ACC
p-mTOR
mTOR
p-S6K
S6K
p-4E-BP
4E-BP
p-PERK
PERK
p-IRE1
IRE1
p-JNK
JNK

Figure 3

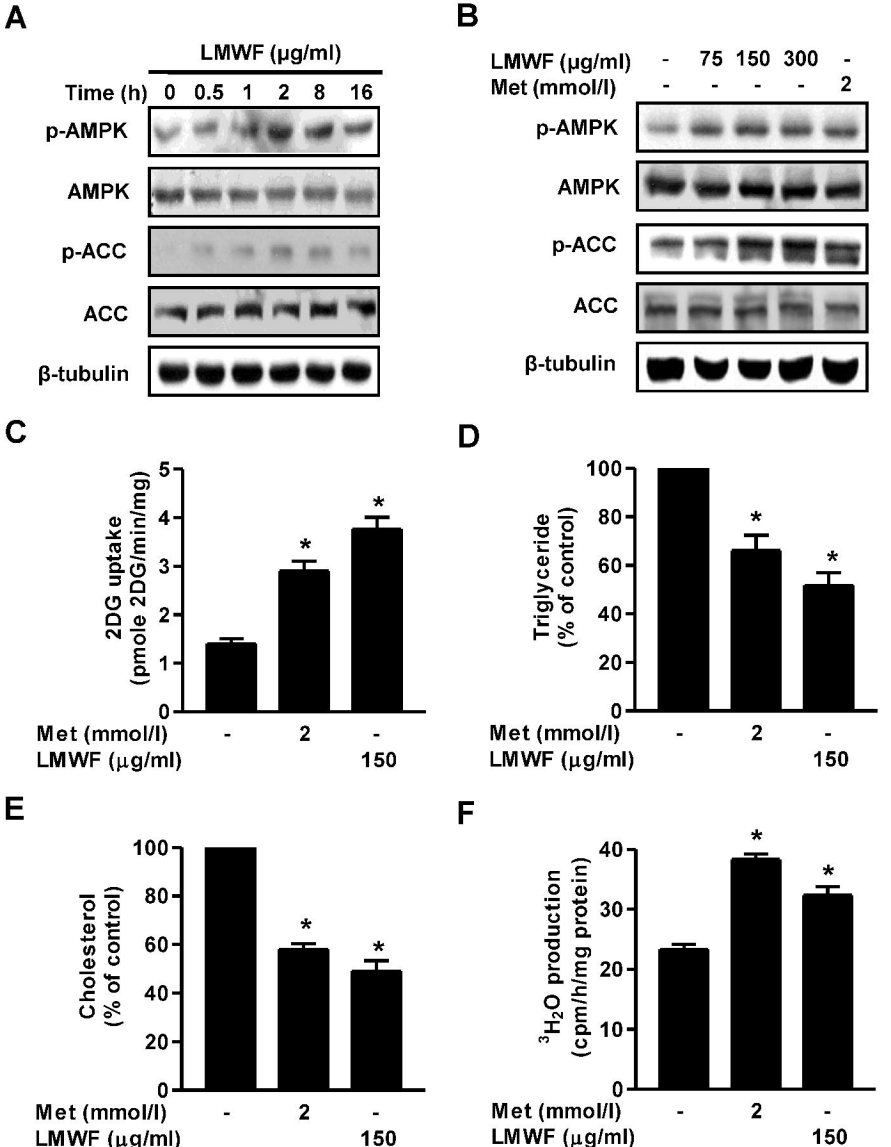

A
LMWF (μg/ml)
Time (h) 0 0.5 1 2 8 16
p-AMPK
AMPK
p-ACC
ACC
β-tubulin
B
LMWF (μg/ml) - 75 150 300 -
Met (mmol/l) - - - - - 2
p-AMPK
AMPK
p-ACC
ACC
β-tubulin
C
2DG uptake (pmole 2DG/min/mg)
0 1 2 3 4 5
Met (mmol/l) - 2 -
LMWF (μg/ml) 150
D
Triglyceride (% of control)
0 20 40 60 80 100
Met (mmol/l) - 2 -
LMWF (μg/ml) 150
E
Cholesterol (% of control)
0 20 40 60 80 100
Met (mmol/l) - 2 -
LMWF (μg/ml) 150
F
^3H_2O production (cpm/h/mg protein)
0 10 20 30 40
Met (mmol/l) - 2 -
LMWF (μg/ml) 150

Figure 4

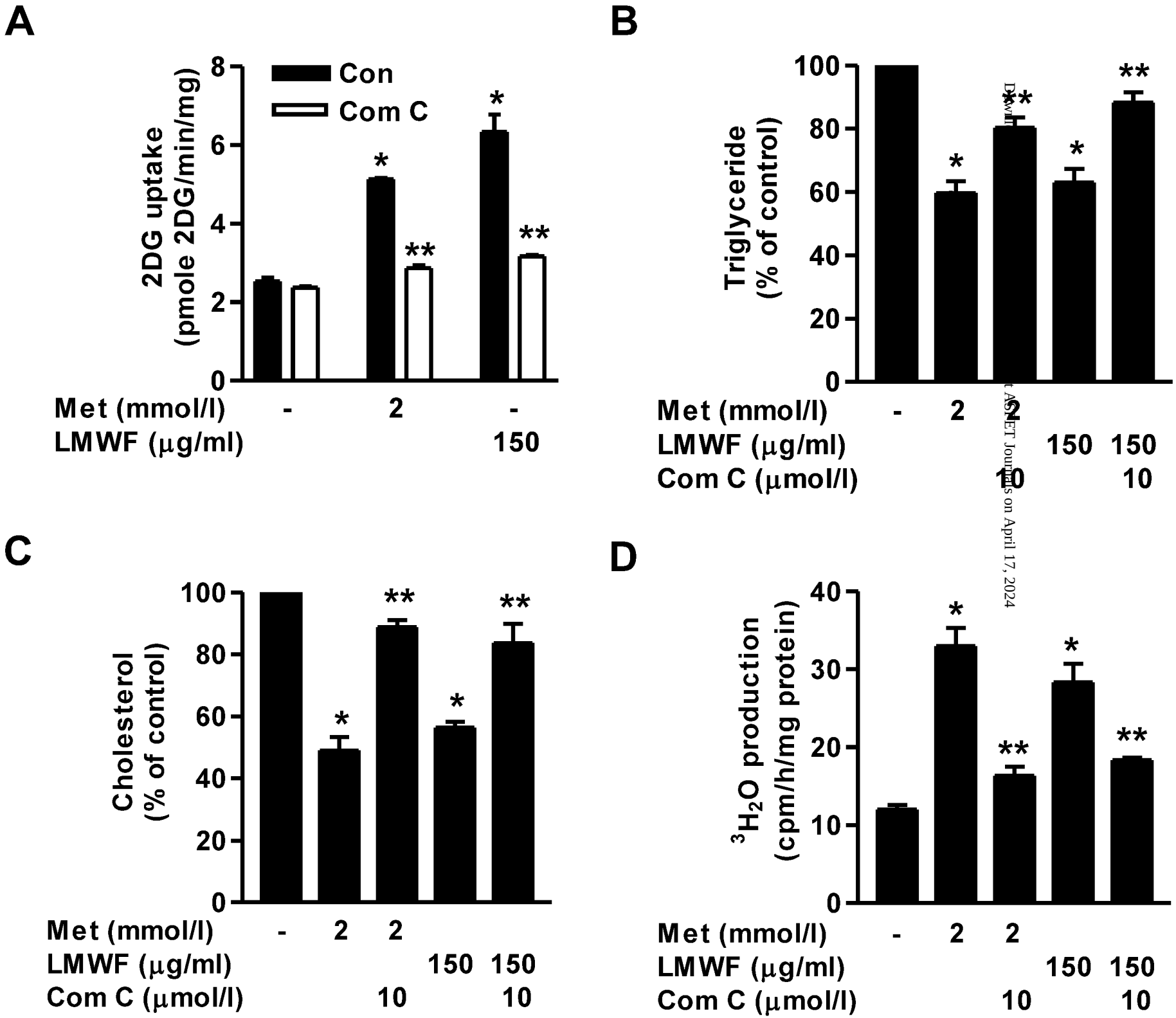


Figure 5

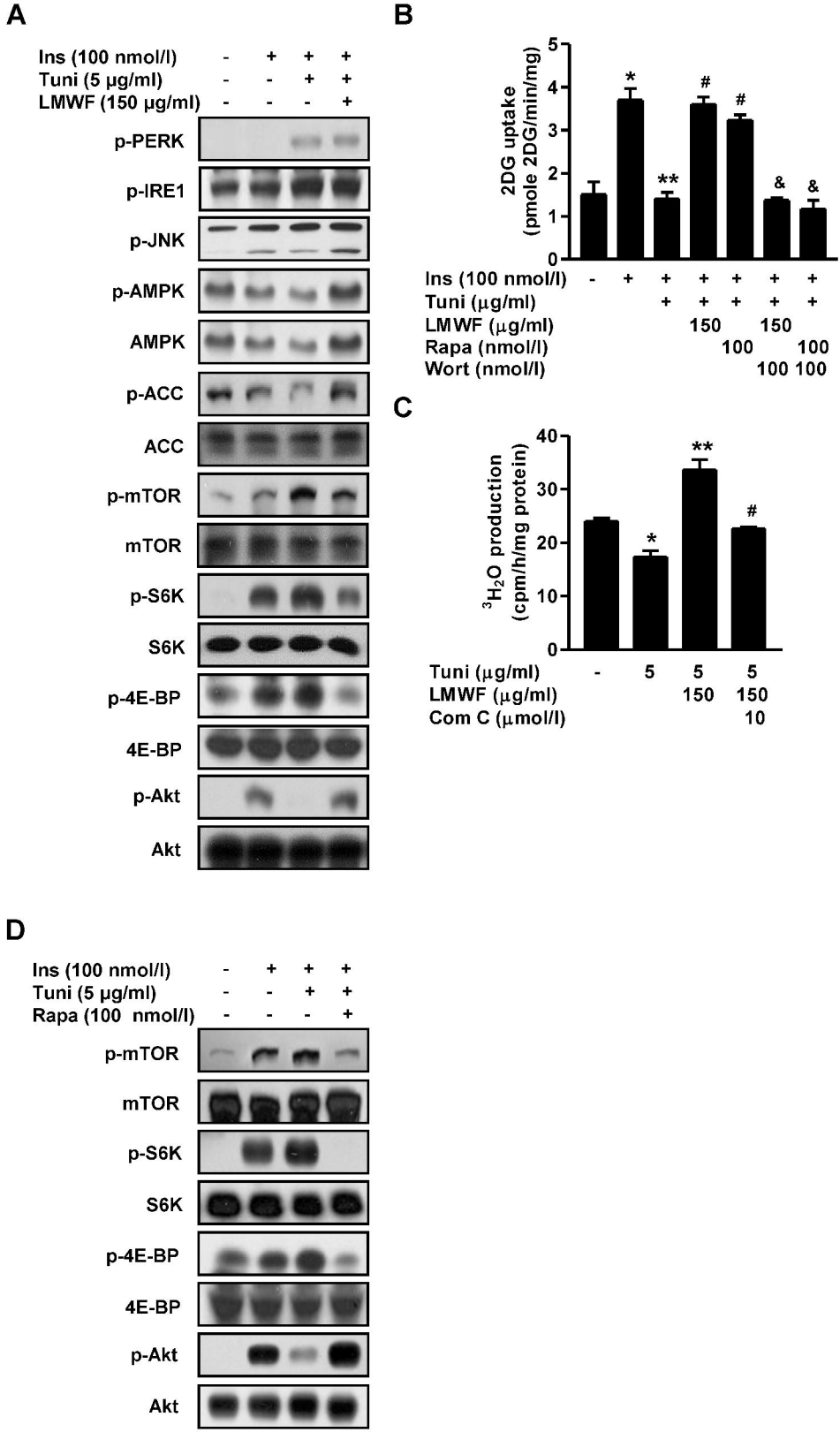
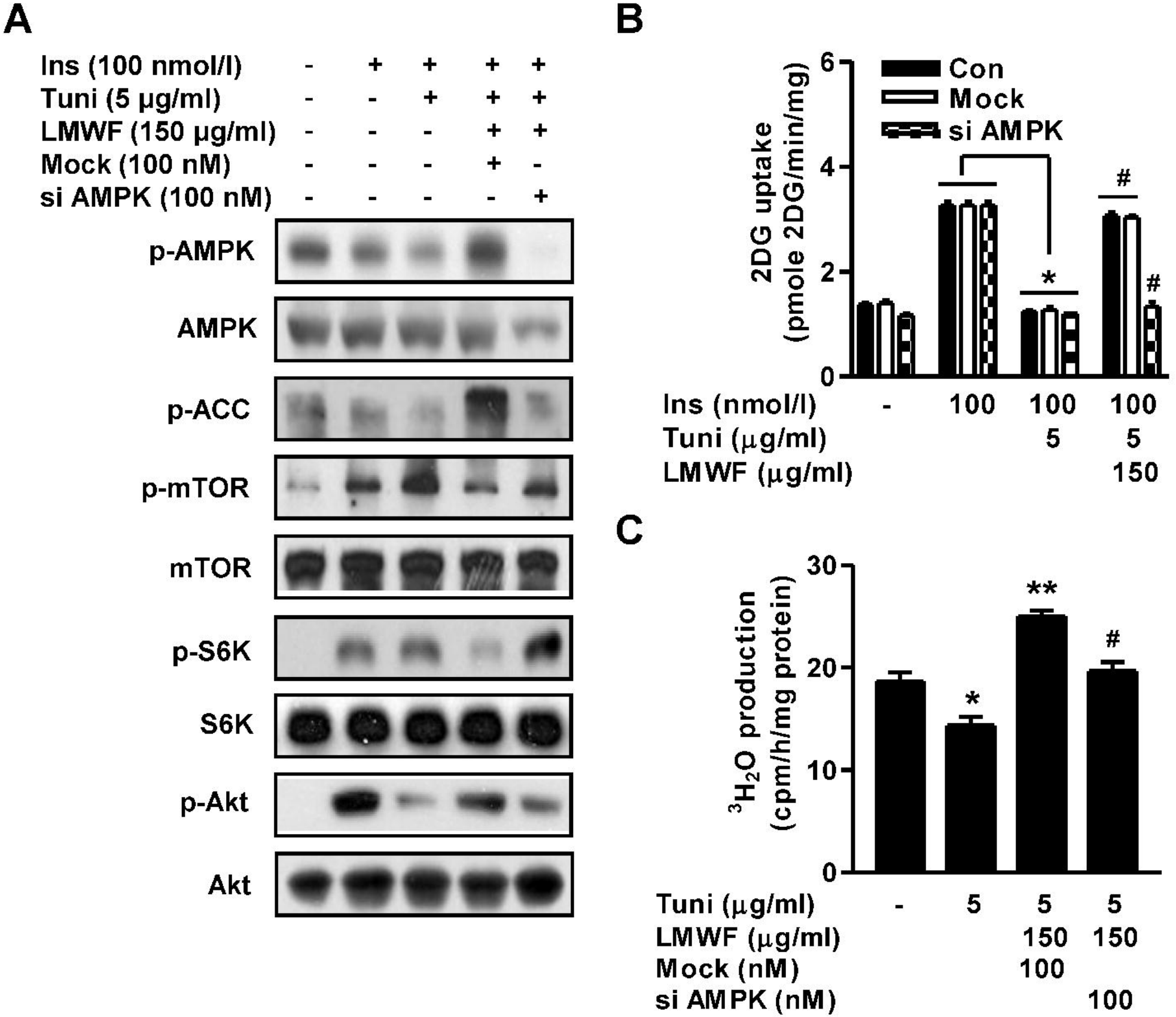

A
Ins (100 nmol/l) - + + +
Tuni (5 μg/ml) - - + +
LMWF (150 μg/ml) - - - +
p-PERK
p-IRE1
p-JNK
p-AMPK
AMPK
p-ACC
ACC
p-mTOR
mTOR
p-S6K
S6K
p-4E-BP
4E-BP
p-Akt
Akt
B
2DG uptake (pmole 2DG/min/mg)
0 1 2 3 4 5
* ** # # & &
Ins (100 nmol/l) - + + + + + +
Tuni (μg/ml) + + + + +
LMWF (μg/ml) 150 150
Rapa (nmol/l) 100 100
Wort (nmol/l) 100 100
C
^3H_2O production (cpm/h/mg protein)
0 10 20 30 40
* ** #
Tuni (μg/ml) - 5 5 5
LMWF (μg/ml) 150 150
Com C (μmol/l) 10
D
Ins (100 nmol/l) - + + +
Tuni (5 μg/ml) - - + +
Rapa (100 nmol/l) - - - +
p-mTOR
mTOR
p-S6K
S6K
p-4E-BP
4E-BP
p-Akt
Akt

Figure 6


A
Ins (100 nmol/l) - + + + +
Tuni (5 μg/ml) - - + + +
LMWF (150 μg/ml) - - - + +
Mock (100 nM) - - - + -
si AMPK (100 nM) - - - - +
p-AMPK
AMPK
p-ACC
p-mTOR
mTOR
p-S6K
S6K
p-Akt
Akt
B
2DG uptake (pmole 2DG/min/mg)
Con
Mock
si AMPK
0
2
4
6
*
#
#
Ins (nmol/l) - 100 100 100
Tuni (μg/ml) 5 5
LMWF (μg/ml) 150
C
^3H_2O production (cpm/h/mg protein)
0
10
20
30
*
**
#
Tuni (μg/ml) - 5 5 5
LMWF (μg/ml) 150 150
Mock (nM) 100
si AMPK (nM) 100

Low molecular weight fucoidan (LMWF) improves ER stress-reduced insulin sensitivity through AMPK activation in L6 myotubes and restores lipid homeostasis in a mouse model of type 2 diabetes

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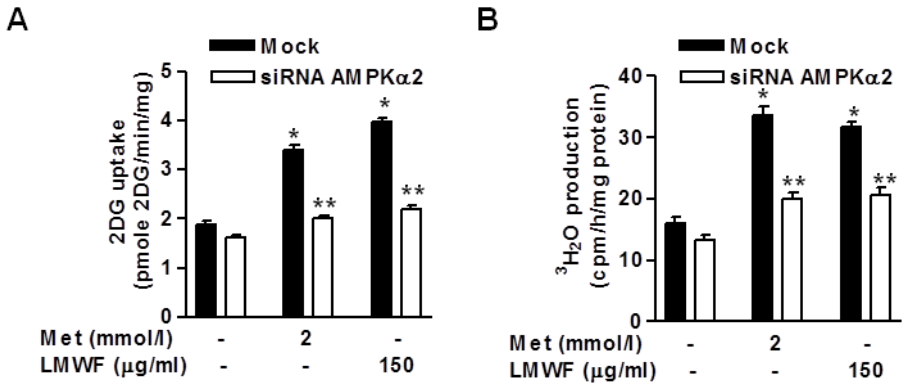
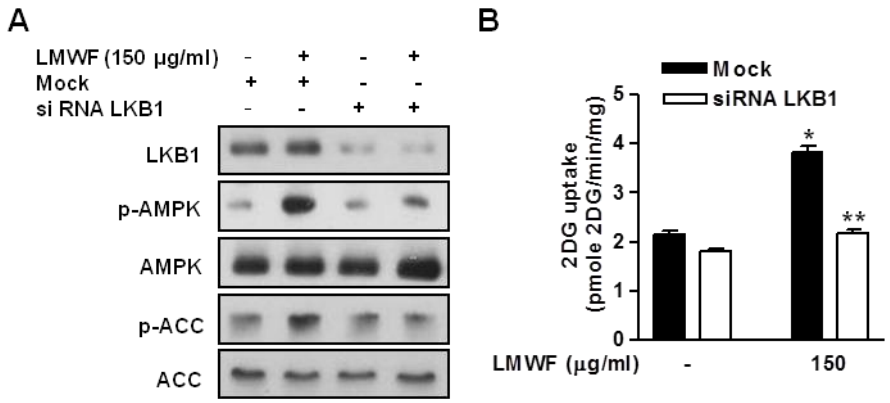
Molecular Pharmacology



Supplemental Figure 1. The effect of LMWF on glucose uptake and fatty acid oxidation is AMPK dependent manner. (A) and (B) L6 myotubes were treated with control siRNA (Mock) or AMPK siRNA and were then treated with LMWF for 2 h (glucose uptake) or 16 h (fatty acid oxidation). Glucose uptake (A) and fatty acid oxidation (B) were measured as described in Methods. All results are representative of at least three independent experiments. ***P* < 0.05 versus untreated controls, ****P* < 0.01 versus LMWF- or metformin-treated cells with control siRNA.



Supplemental Figure 2. Stimulation of AMPK activity and glucose uptake by LMWF was mediated by LKB1. (A) L6 myotubes were infected with control siRNA (Mock) or LKB1 siRNA for 48 h. Cells were then treated with LMWF for 2 h. Whole cell extracts were isolated and analyzed by Western blotting using indicated antibodies. Protein production was normalized with respect to non-phosphorylated forms (total forms). (B) Under the same conditions, glucose uptake was measured as described in Methods. All results are representative of at least three independent experiments. ***P* < 0.05 versus untreated controls, ****P* < 0.01 versus LMWF-treated cells with control siRNA.