Low Molecular Weight Fucoidan Improves Endoplasmic Reticulum Stress-Reduced Insulin Sensitivity through AMP-Activated Protein Kinase Activation in L6 Myotubes and Restores Lipid Homeostasis in a Mouse Model of Type 2 Diabetes

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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 obese diabetic mice (leptin receptor−deficient 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 low-density lipoprotein levels, and gradually reduced body weights by reducing lipid parameters. Furthermore, it effectively ameliorated glucose homeostasis by elevating glucose tolerance. In addition, the phosphorylation levels 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 the mammalian target of rapamycin–p70S61 kinase 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.

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 (Hayashi et al., 2008; Kim et al., 2012). Moreover, recent studies have shown that low molecular weight fucoidan (LMWF) reduces lipid accumulation by retarding adipogenesis in 3T3-L1 cells (Park et al., 2011). However, although fucoidan is known to regulate many physiologic functions (Cumashi et al., 2007), 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 demonstrated that genetic and dietary obesity are associated with ER stress (Ozcan et al., 2004; Wellen and Hotamisligil, 2005) occurring secondary to increased mammalian target of rapamycin (mTOR) activity and increased 4E-BP phosphorylation (Ozcan et al., 2004). In this review, we will address the relationship between mTOR activity and ER stress, and how fucoidan might act as a therapeutic strategy for the treatment of metabolic diseases.
protein synthesis (Ozcan et al., 2008). The conserved serine/threonine kinase mTOR 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, we showed that ER stress-mediated mTOR activation is associated with reduced insulin signaling in muscle cells (Hwang et al., 2012), and a direct link between mTOR/p70S6 kinase (S6K) activation and repressed insulin action was suggested to be caused by the reversal of this effect by rapamycin (a mTOR inhibitor) in L6 myotubes (Hwang et al., 2012). These observations demonstrate that ER stress plays a significant role in the mTOR/S6K-mediated negative-feedback inhibition of insulin activity.

AMPK-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 (Hardie et al., 2003; Carling, 2004). AMPK phosphorylates its downstream substrates and thus reduces ATP-consuming anabolic pathways, such as those responsible for the synthesis of fatty acids, cholesterol, and triacylglycerols, and increases ATP-generating catabolic pathways, such as fatty acid oxidation and lipolysis (Hardie et al., 2003; Iglesias et al., 2004). 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 (Merrill et al., 1997; Mu et al., 2001; Aschenbach et al., 2004). Furthermore, it was demonstrated that the activation of AMPK suppresses oxidized low-density lipoprotein (LDL)–induced ER stress by inhibiting NAD(P)H oxidase–derived reactive oxygen species production and the sarco/endoplasmic reticulum Ca-ATPase 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 (leptin receptor–deficient db/db mice), LMWF ameliorated hyperlipidemia and insulin resistance in vivo. Strikingly, LMWF reduced ER stress in the muscle tissue of db/db mice as well as in tunicamycin-incubated L6 myotubes and improved insulin signaling. Based on these results, it appears that LMWF activates AMPK and alleviates ER stress, making it a potentially new treatment of metabolic dysfunctions.

**Materials and Methods**

Tunicamycin (Hwang et al., 2012), 2-deoxyglucose, compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine) (Zhou et al., 2001), wortmannin, and rapamycin (Hwang et al., 2008) were purchased from Calbiochem (Darmstadt, Germany). Metformin (Bailey and Turner, 1996), insulin, bovine serum albumin, a-pyrimidine (Zhou et al., 2001), wortmannin, and rapamycin (Hwang et al., 2001; Aschenbach et al., 2004). Furthermore, it was demonstrated that the activation of AMPK suppresses oxidized low-density lipoprotein (LDL)–induced ER stress by inhibiting NAD(P)H oxidase–derived reactive oxygen species production and the sarco/endoplasmic reticulum Ca-ATPase 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.

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

**Animal Experimental Procedures.** Male C57BL/Kej-Leprdb/db (db/db) mice and lean C57BL/6Jms (wild-type or Lean) mice purchased from the Jackson Laboratories (Bar Harbor, ME) were housed in a temperature-controlled room (22°C ± 2°C) under a 12-hour 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 high-density lipoprotein (HDL), LDL, triglyceride, and total cholesterol levels were measured in blood samples or tissues as appropriate. The Institutional Animal Use and Care Committee of Yeungnam University approved all animal studies and the study protocol.

**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-hour fast. Blood glucose was measured at the indicated times. All blood samples were collected from mice fasted for 12 hours and plasma samples were immediately prepared. Triglycerides, total cholesterol, LDL, and HDL levels were measured using an automated blood analyzer (AU400; Olympus, Tokyo, Japan), and adiponectin and leptin levels were detected using a commercially available enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) or a radioimmunoassay kit (Linco Research, St. Charles, MO), respectively, as described previously (Sahai et al., 2004; Fujita et al., 2005).

**Histologic Analysis.** Histologic analysis was performed as described previously (Hoe et al., 2004; Villena et al., 2004). Briefly, mice were sacrificed 6 weeks after administering LMWF (250 or 500 mg/kg) and/or metformin (the positive control; 250 mg/kg). Liver tissues were collected by perfusion, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 4 μm, and stained with Oil Red O. Cell size was measured following DakoCytoMation (Dako, Carpinteria, CA) according to the manufacturer’s instructions.

**Cell Culture.** L6 cells (American Type Culture Collection, Manassas, VA) were maintained in α-minimum Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO2 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 (micrograms of lipid per milligram of cellular protein) are expressed as percentages of nontreated controls. In brief, L6 myotubes were maintained in serum-free medium overnight and incubated for 16 hours in the absence or presence of 150 μg/ml LMWF. Cell lysates were prepared as described above. Triglycerides and total cholesterol levels in cell lysates were measured using Infinity reagents (Thermo Dyna, Louisville, CO), according to the manufacturer’s instructions.

**Fatty Acid Oxidation.** Palmitate oxidation was analyzed as described previously (Cabrero et al., 2001).

**Immunoblotting.** L6 myotubes and tissue samples were isolated and processed as described previously (Hwang et al., 2008). The polyvinylidene difluoride membranes (Calbiochem) were probed with liver kinase B1 (LKB1), AMPK, pAMPK, ACC (acetyl-CoA carboxylase), pACC, protein kinase-like ER resident kinase (PERK), pPERK, inositol-requiring kinase-1 (IRE1), pIRE1, c-Jun NH2-terminal kinase (JNK), pJNK, mTOR, pmTOR, S6K, pS6K, 4E binding protein (4E-BP), p4E-BP, Akt, and pAkt (all from Cell Signaling Technology, Beverly, MA), and β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and then developed using an enhanced chemiluminescence Western blot detection kit (Amersham Bioscience, Piscataway, NJ).

**Transfection with Small Interfering RNA.** For small interfering RNA (siRNA) experiments, SMARTpool for rat LKB1 (L-10059- 01-0020) and rat AMPKα2 (L-100623-00-0020) was obtained from Dharmacon (Lafayette, CO). Nonspecific siRNA (Control siRNA, South Korea).
D-001810-10-20; Thermo Scientific, Pittsburgh PA) was used as a control. For transient expression experiments, L6 myotubes were serum-starved for 16 hours in serum-free media, and then transfected with DharmaFECT transfection reagent (Dharmacon) in 12- or 60-mm plates containing 100 nM of either LKB1 siRNA, AMPKα2 siRNA, or nontargeting control siRNA per plate according to the manufacturer’s protocol. After 48 hours, cells were treated with vehicle or LMWF for 2 hours, then with tunicamycin (5 μg/ml) for 3 hours, and finally, with insulin (100 nM) for 10 minutes. Cells were then subjected to immunoblot or glucose uptake experiments.

Statistical Analysis. Data are expressed as mean ± S.E.M. Analysis of variance and/or the t test was used to determine the significances of differences. P values <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 or 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 was not significantly different (Fig. 1, A and B). As shown in Fig. 1C, gross observations of LMWF-treated mice showed significantly less white adipose tissue and more small adipocytes in epididymis fat pad than in untreated db/db mice. Furthermore, the abdominal adipocytes of untreated db/db mice exhibited severe hypertrophy 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. 1, E and 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 physiologic role of LMWF on the regulations of glucose metabolism and adipokine levels, LMWF (250 or 500 mg/kg) or metformin (250 mg/kg) was administered once to db/db mice. As shown in Fig. 2, A and B, LMWF gradually reduced blood glucose levels and significantly improved glucose tolerance 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 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 (Weyer et al., 2001; Cnop et al., 2003). As we 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 in 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) is affected by LMWF in diabetic mice. As we expected, LMWF significantly elevated the AMPK-ACC axis compared with untreated controls, and subsequently dose-dependently depressed the mTOR, S6K, 4E-BP, PERK, IRE1, 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 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. 3, D and E). 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 we 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 investigate the effects of LMWF on AMPK activity, we treated L6 myotubes with compound 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 compound C (Fig. 4A). Furthermore, LMWF or metformin also markedly reduced triglyceride and cholesterol contents in L6 myotubes, and these inhibitory effects were prevented by compound C (Fig. 4, B and C). In addition, LMWF or metformin significantly elevated fatty acid oxidation, and this was markedly reduced by pretreated with compound 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 regulates AMPK activity (Sakamoto et al., 2005). We also attempted to reduce LKB1 expression using its specific siRNA to ascertain the contribution of this master kinase to AMPK activation. 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 effect 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 showed that tunicamycin promotes the activations of ER stress markers, such as RNA-activated PERK, IRE1, JNK, and mTOR-S6K, and that these activations reduce insulin signaling (Ozcan et al., 2004, 2006; Um et al., 2004). Based on these findings,
Fig. 2. LMWF improved glucose metabolism and adipokine profiles in db/db mice. Mice were treated for 6 weeks with LMWF (250 or 500 mg/kg) or metformin (250 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), and adiponectin levels (E). (F) Effect of LMWF on adiponectin levels in adipose tissues. (G) Under the same conditions, whole cell extracts were isolated from skeletal muscle and analyzed by Western blotting with various antibodies, and then normalized versus nonphosphorylated forms (total forms). Metformin (250 mg/kg) was used as a positive control. Experimental groups were composed of 5–7 mice. *P < 0.05 versus untreated db/db mice. M, metformin; WT, wild-type.
we investigated the relation between ER stress and the action of insulin in L6 myotubes. ER stress-dependent signal pathways, such as PERK, IRE1, JNK, and mTOR-S6K were markedly increased by tunicamycin 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 lower by LMWF (Fig. 5C). However, LMWF decreased the activations of PERK, IRE1, and JNK in muscle tissues from db/db mice (Fig. 2G), it had no modulatory effect on the activations of PERK, IRE1, 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 after LMWF treatment. 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 we expected, AMPKα2 siRNA significantly decreased the protein levels and the activity of α2 AMPK (Fig. 6A). Furthermore, siRNA silencing of AMPKα2 reduced...
the LMWF-induced phosphorylation of AMPK (Fig. 6A). Moreover, AMPKα2 siRNA completely abolished LMWF preserved insulin-stimulated Akt phosphorylation and glucose uptake under ER stress (Fig. 6, A and 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 phosphorylation of insulin receptor substrate-1 by mTOR-S6K was recently shown to interrupt the interaction between insulin receptor substrate-1 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 upregulation of AMPK by LMWF promotes insulin sensitivity by controlling an ER-stress–mediated signaling pathway.

The physiologic 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 in db/db mice within 14–42 days after initiating LMWF treatment, and lipid parameters, fat weights, and serum leptin and triglyceride levels were dramatically decreased (Figs. 1 and 2). Previous studies demonstrated that excessive adipose tissue leads to increased fat accumulation and adipose cell hypertrophy and that it reduces adiponectin levels in obese rodents and humans (Arita et al., 1999; Morange et al., 2000). 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 that LMWF might be effective at ameliorating metabolic abnormalities. While our studies strongly suggest that LMWF improves glucose and lipid profiles via AMPK in db/db mice, future experiments with AMPK-deficient mice will be required to confirm this.

**Discussion**

LMWF was 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.
ER stress is receiving more attention because it has been linked to the pathogenesis of type 2 diabetes and to the inhibition of insulin signaling (Ozcan et al., 2004, 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 (Figs. 3 and 4). Plasma concentrations of LMWF measured using liquid chromatography coupled to tandem mass spectrometry at steady state range from 18 to 30 mg/ml and from 38 to 46 mg/ml at doses of 250 and 500 mg/kg, respectively, in mice (unpublished data). The concentrations (150 mg/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 [area under the plasma level-time curve (AUC), approximately 576 mg·h/ml at the dose of 250 mg/kg], which represents the systemic exposure of the compound in mice after 6-week once-daily repeated oral administration.

As mentioned above, tunicamycin was found to stimulate ER stress markers, PERK, IRE1, JNK, mTOR, and S6K and to repress insulin-stimulated Akt activation and glucose uptake (Figs. 5, A and B). On the other hand, LMWF enhanced the action of insulin and reduced 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 downregulate insulin-stimulated Akt phosphorylation and glucose uptake (Tzatsos and Kandror, 2006), these results suggest that LMWF improves insulin sensitivity by downregulating the S6K-dependent pathway, which is consistent with the beneficial effects of LMWF in muscle cells. In skeletal muscle, AMPK directly phosphorlyates at least two proteins and rapidly suppresses the activities of mTORC1 (a tuberous sclerosis complex 2 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 PI3K activity, AMPK activation actually serves to attenuate this feedback and to promote autonomous restoration of IRS protein levels in cells and IRS signaling to PI3K. 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 high, IRS protein levels are low and Akt is inhibited; however, 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 (Saltiel and Kahn, 2001; Krook et al., 2004) importantly regulate glucose homeostasis in muscle cells. Activated insulin receptor transduces insulin action by stimulating the PI3K-Akt pathway, and subsequently promotes glucose uptake in muscle cells. In a previous study, we attempted to ameliorate insulin resistance by controlling the upregulation 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 AMP-ACC pathway in L6 myotubes, our findings show that LMWF enhances insulin sensitivity by stimulating an AMPK-dependent pathway, which concurs with previous reports (Merrill et al., 1997; Iglesias et al., 2004). Furthermore, we found that by controlling this AMPK-dependent pathway in L6 myotubes, LMWF reduced intracellular triglyceride and cholesterol levels (Figs. 3 and 4). Thus, our findings suggest that LMWF reduces lipid levels in L6 myotubes, which is consistent with the stimulation of the AMP-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 phosphorylation of AMPK in AMPK knockout mice, and 2) what extent the lipid-lowering effects of LMWF are due to 1) reducing lipid levels in L6 myotubes, which is consistent with previous reports (Merrill et al., 1997; Iglesias et al., 2004). Furthermore, our findings show that LMWF enhances insulin sensitivity, 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 into 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.

Authorship Contributions

**Participated in research design:** Jeong, Y. D. Kim, Hwang.
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**Contributed new reagents or analytic tools:** Ku, Baek, Kang.
**Performed data analysis:** Y. D. Kim, Hwang, Chang.
**Wrote or contributed to the writing of the manuscript:** Y. D. Kim, Hwang, Chang.

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Hong F, Radaeva S, Pan HN, Tian Z, Veech R, and Gao B (2004) Interleukin 6 reduces lipid levels in L6 myotubes, which is consistent with a previous report (Merrill et al., 1997; Iglesias et al., 2004). Furthermore, our findings show that LMWF enhances insulin sensitivity, 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 into 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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