ORIGINAL ARTICLE

Therapeutic Potential of Peroxisome Proliferators–Activated Receptor-α/γ Dual Agonist With Alleviation of Endoplasmic Reticulum Stress for the Treatment of Diabetes

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OBJECTIVE—Peroxisome proliferator–activated receptor (PPAR) α/γ dual agonists have the potential to be used as therapeutic agents for the treatment of type 2 diabetes. This study evaluated the function of macelignan, a natural compound isolated from *Myristica fragrans*, as a dual agonist for PPARα/γ and investigated its antidiabetes effects in animal models.

RESEARCH DESIGN AND METHODS—GAL4/PPAR chimera transactivation was performed and the expression of PPARα/γ target genes was monitored to examine the ability of macelignan to activate PPARα/γ. Additionally, macelignan was administrated to obese diabetic (*db/db*) mice to investigate antidiabetes effects and elucidate its molecular mechanisms.

RESULTS—Macelignan reduced serum glucose, insulin, triglycerides, free fatty acid levels, and triglycerides levels in the skeletal muscle and liver of *db/db* mice. Furthermore, macelignan significantly improved glucose and insulin tolerance in these mice, and without altering food intake, their body weights were slightly reduced while weights of troglitazone-treated mice increased. Macelignan increased adiponectin expression in adipose tissue and serum, whereas the expression and serum levels of tumor necrosis factor-α and interleukin-6 decreased. Macelignan downregulated inflammatory gene expression in the liver and increased AMP-activated protein kinase activation in the skeletal muscle of *db/db* mice. Strikingly, macelignan reduced endoplasmic reticulum (ER) stress and c-Jun NH_2-terminal kinase activation in the liver and adipose tissue of *db/db* mice and subsequently increased insulin signaling.

CONCLUSIONS—Macelignan enhanced insulin sensitivity and improved lipid metabolic disorders by activating PPARα/γ and attenuating ER stress, suggesting that it has potential as an antidiabetes agent for the treatment of type 2 diabetes. ***Diabetes* 57:737–745, 2008**

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ACO, acyl-CoA oxidase; AMPK, AMP-activated protein kinase; aP2, adipose fatty acid–binding protein; CPT, carnitine palmitoyltransferase; ER, endoplasmic reticulum; FFA, free fatty acid; IκBα, inhibitor of κBα; IL, interleukin; IRS, insulin receptor substrate; JNK, c-Jun NH_2-terminal kinase; LPL, lipoprotein lipase; NF, nuclear factor; PPAR, peroxisome proliferators–activated receptor; PPRE, PPAR response element; TNF, tumor necrosis factor; UCP, uncoupling protein.

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The worldwide prevalence of type 2 diabetes is steadily rising; therefore, in addition to a more aggressive approach in managing diabetes through diet and exercise, antidiabetes agents that ameliorate insulin resistance and hyperlipidemia are also needed.

As members of the nuclear hormone receptor superfamily, peroxisome proliferator–activated receptors (PPARs) bind to specific DNA response elements as heterodimers with the retinoid-X receptor to control glucose and lipid metabolism, which offers a promising therapeutic approach for treating the metabolic syndrome (1). There are several PPAR isoforms, including PPAR α, γ, and δ that share 60–80% homology in the ligand- and DNA-binding domains (2). Widely expressed in the liver, PPARα functions in the catabolism of fatty acids responsible for decreasing serum triglyceride levels and increasing HDL cholesterol levels in dyslipidemia (3). Therefore, PPARα agonists have the potential to be used to ameliorate insulin resistance and hyperlipidemia. Moreover, PPARγ is highly expressed in adipocytes and is involved in adipocyte differentiation, lipid storage, glucose homeostasis, and adipocytokine regulation, which can improve insulin sensitivity and glucose tolerance (4). The primary issue with the utility of classic full PPARγ agonists is that they exert a variety of side effects, chiefly weight gain due to edema and increased fat mass (5). However, the side effects associated with PPARγ activation may be circumvented through the combined activation of PPARα and -γ, which is known to result in a complementary and synergistic increase in lipid metabolism and insulin sensitivity (6). As such, pharmaceutical trials are ongoing to develop dual PPARα/γ agonists that possess the beneficial metabolic effects of PPARα and -γ activation but with fewer side effects.

Natural products are a rich source of ligands for nuclear receptors and are promising therapeutic agents in clinical practice. In particular, plants provide an abundant source of biologically active molecules that have played critical roles in pharmacology. Based on a history of traditional therapeutic applications, many of these natural products have demonstrated beneficial medicinal attributes. For example, the pharmaceutical antidiabetes agent, metformin, is a derivative of natural plant products (7).

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Exploring the use of natural products to treat type 2 diabetes has not previously been studied in depth. However, the recent identification of biological compounds derived from plants that are capable of modulating PPARα/γ has received much attention. The *Myristica fragrans* Houtt plant, also commonly known as nutmeg or mace, has been used traditionally as a spice and for medicinal purposes in several Asian countries (8–10).

In this study, we examined extracts of *Myristica fragrans* Houtt to search for a natural PPARα/γ dual agonist and found that macelignan compound activated both PPARα and -γ. When the antidiabetes effects of macelignan were explored in obese diabetic mice (*db/db* mice), macelignan ameliorated insulin resistance and hyperglycemia in vivo. Strikingly, macelignan reduced endoplasmic reticulum (ER) stress in the liver and white adipose tissue of *db/db* mice as well as in thapsigargin-incubated SK-HEP1 and 3T3-L1 cells and improved insulin receptor substrate (IRS)-1 signaling. Based on these results, it appears that macelignan activates both PPARα and -γ, and alleviates ER stress, making it a potentially new treatment for type 2 diabetes.

RESEARCH DESIGN AND METHODS

Plant materials. *Myristica fragrans* Houtt (Myristicaceae) was obtained from the Biofarmaka Research Center of Bogor Agricultural University (Indonesia). The active compound identified as macelignan (Fig. 1*A*) was isolated from the extract of *Myristica fragrans* Houtt (Myristicaceae) as described previously (11).

Cells. COS-7 cells, 3T3-L1 preadipocytes, SK-HEP1 hepatocytes, HepG2 hepatocytes, and C2C12 skeletal myoblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The 3T3-L1 preadipocytes were differentiated as previously described (12). C2C12 skeletal myoblast cells were grown in Dulbecco's modified Eagle's medium supplemented with 2% horse serum to induce differentiation into myotubes.

Animals. The care and experimentation of animals in this investigation were conducted according to good welfare protocols in the *Guide for Care and Use of Laboratory Animals* at the National Institutes of Health of Korea. Obese diabetic mice (*db/db*) and age-matched lean normal C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in polycarbonate cages in a temperature-regulated (22°C) and humidity-controlled (55%) room with a 12-h light/12-h dark cycle. Water and a normal standard pellet diet were available ad libitum throughout the experimental period. Animals were killed after a 6-h fast. Liver, soleus skeletal muscle, and gonadal fat pads were collected and stored at −70°C for further analysis.

GAL4/PPAR chimera assay and reporter gene assay. The PPAR ligand-binding activity was measured using a GAL4/PPAR chimera assay as described previously (13). Plasmids containing three PPAR response elements (PPREs), PPRE-tk-Luc, were a gift from Dr. R.M. Evans (Howard Hughes Medical Institute, Los Angeles, CA). After HepG2 or 3T3-L1 cells (2×10^5 cells/well) were transfected with PPRE-tk-Luc, cells were incubated with macelignan, troglitazone (Sigma, St. Louis, MO), or WY14643 (Sigma) at doses ranging from 0.01 to 25 μmol/l for 24 h. Luciferase activities were then determined. The EC_{50} values were calculated by GraphPad Prism version 4.0 C.

Real-time RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and subjected to reverse transcription using reverse transcriptase (Promega, Madison, WI) then subsequently amplified by PCR using gene-specific primers. To quantify mRNA expression, PCR was performed using a fluorescence temperature cycler (Chromo4, Real-Time PCR System; Bio-Rad, Hercules, CA).

Western blot analysis and immunoprecipitation. Total proteins were extracted using PRO-PREP reagent (Intron Biotechnology, Sungnam, Korea), and immune complexes were identified using the enhanced chemiluminescence detection system (Amersham Biosciences, Uppsala, Sweden). To detect tyrosine or serine phosphorylated IRS-1, total proteins were immunoprecipitated with anti–IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody and then subjected to Western blot analysis with anti-phosphoserine (307) IRS-1 (Cell Signaling, Beverly, MA) or anti-phosphotyrosine (Santa Cruz Biotechnology).

Biochemical analysis. Serum triglycerides, free fatty acids (FFAs) and HDL cholesterol levels were determined using commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). Serum insulin levels were measured with the insulin–enzyme immunosorbent assay test kit (Shibayagi, Gunma, Japan). Serum adiponectin, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 levels were determined by mouse enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). Triglyceride levels in tissue or adipocytes were also measured with commercial kits (Wako Pure Chemical Industries) after lipid extraction. β-Oxidation was measured as described previously (14). The activity was determined by counting the radioactivity and expressed as relative activity compared with untreated *db/db* mice.

Glucose tolerance test and insulin tolerance test. For the glucose tolerance test, animals were administered macelignan or troglitazone orally for 14 days and then challenged with oral glucose (3 g/kg body wt) after fasting 12 h. Blood samples were collected at 0, 30, 60, and 120 min after glucose load. For the insulin tolerance test, mice were fasted for 12 h and injected intraperitoneally with regular human insulin (2.0 units/kg body wt) and blood samples collected at various times. Blood glucose was measured using a portable glucose meter (Glucocard II Arkray, Kyoto, Japan).

Statistical analysis. All data were expressed as means ± SD. One-way ANOVA was used to determine statistically significant differences between groups. Scheffé's test (SPSS12.0; SPSS, Chicago, IL) was used to correct for multiple comparisons when statistical significances were identified in the ANOVA. The EC_{50} values were calculated using GraphPad Prism version 4.0c (GraphPad Software, San Diego, CA).

RESULTS

Function of macelignan as a PPARα/γ dual agonist. The structure of macelignan is shown in Fig. 1*A*. Using cell-based GAL4/PPAR chimera transactivation assays, we investigated whether macelignan acts as a dual agonist for both PPARα and -γ. As shown in Fig. 1*B*, macelignan increased PPARγ-dependent luciferase activity with an EC_{50} of 4.221, whereas troglitazone, a well-known PPARγ agonist, increased PPARγ transactivation with an EC_{50} of 0.214, showing that, comparatively, macelignan is less potent than troglitazone. To further explore the PPARγ agonist potential of macelignan, transient transfections were performed in differentiated 3T3-L1 adipocytes using the tk-luciferase vector containing three PPAR-responsive elements (3 × PPREs) and treated with macelignan. Treatment with macelignan stimulated PPRE-dependent luciferase activities in transfected cells (Fig. 1*C*), but the activity exhibited was lower than troglitazone. To provide biological evidence that macelignan is a PPARγ ligand, we investigated adipocyte differentiation and expression of adipocyte marker genes in macelignan-treated 3T3-L1 cells. Macelignan treatment significantly induced formation of lipid droplets (Fig. 1*D*, *upper*). Although fewer lipid droplets were formed after macelignan treatment than troglitazone treatment, their presence is indicative of 3T3-L1 cell differentiation and is consistent with increased expression of adipocyte marker genes such as adipose fatty acid–binding protein (aP2), fatty acid synthase, PPARγ, and CCAAT/enhancer binding protein α (Fig. 1*D*, *lower left panel*). When the amount of triglycerides was examined during differentiation, the amount found in macelignan-treated cells was also increased compared with untreated cells but was less than troglitazone-treated cells after 7 days (Fig. 1*D*, *lower right panel*), further indicating that macelignan is less adipogenic than troglitazone in 3T3-L1 cells. Moreover, macelignan increased expression of PPARγ target genes such as lipoprotein lipase (LPL), fatty acid synthase, aP2, and GLUT4 in 3T3-L1 adipocyte cells (Fig. 1*E*). Collectively, these results demonstrate that macelignan definitely activates PPARγ, but this activity is lower than the full PPARγ agonist troglitazone. Macelignan was also found to activate the PPARα isoform at an EC_{50} of 5.405 in PPARα/GAL4 chimera transactivation assays (Fig. 1*F*), which was confirmed in HepG2 cells transiently transfected with the (3 ×

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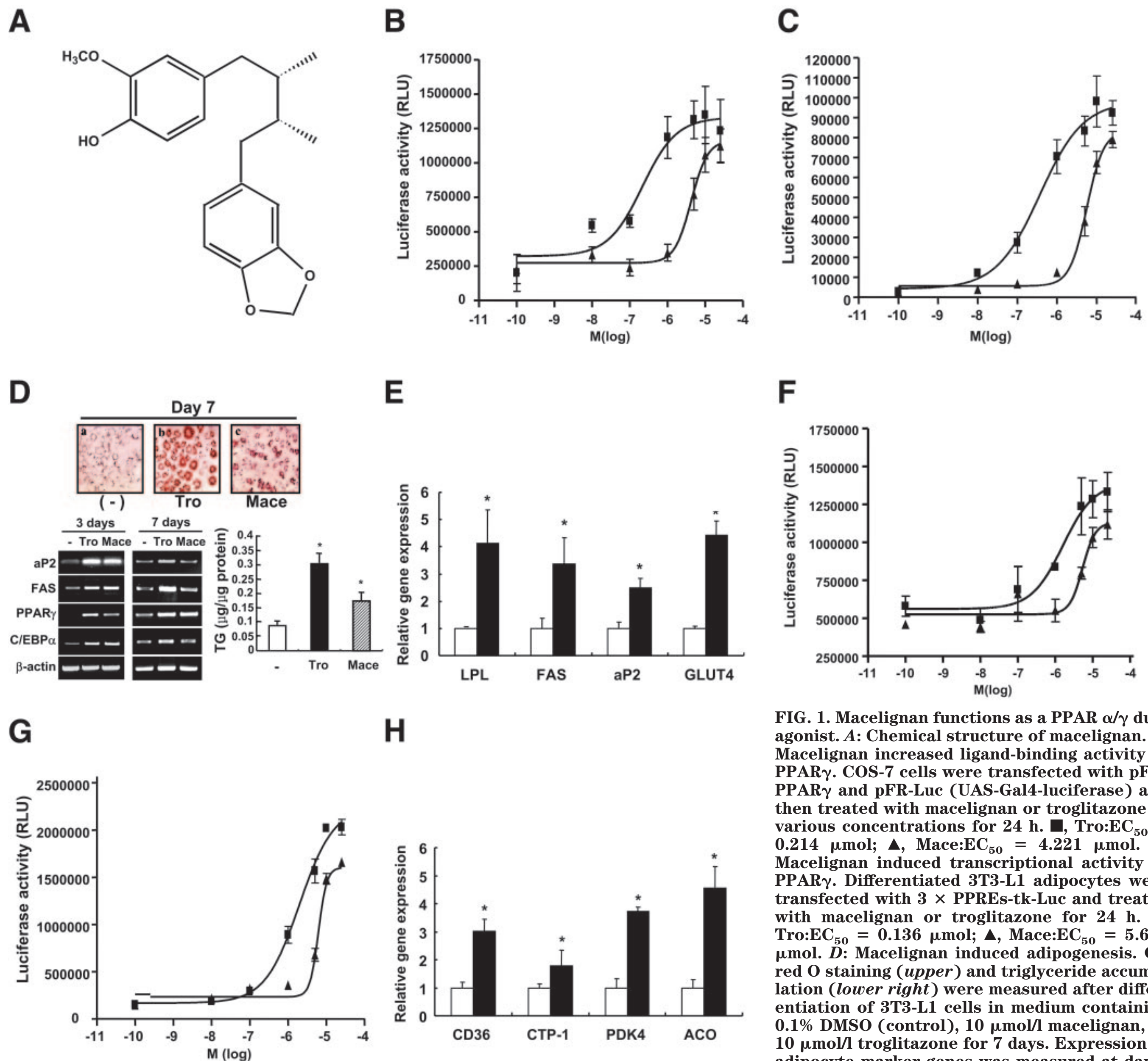

FIG. 1. Macelignan functions as a PPAR α/γ dual agonist. *A*: Chemical structure of macelignan. *B*: Macelignan increased ligand-binding activity of PPARγ. COS-7 cells were transfected with pFA-PPARγ and pFR-Luc (UAS-Gal4-luciferase) and then treated with macelignan or troglitazone at various concentrations for 24 h. ■, Tro:EC_{50} = 0.214 μmol; ▲, Mace:EC_{50} = 4.221 μmol. *C*: Macelignan induced transcriptional activity of PPARγ. Differentiated 3T3-L1 adipocytes were transfected with 3 × PPREs-tk-Luc and treated with macelignan or troglitazone for 24 h. ■, Tro:EC_{50} = 0.136 μmol; ▲, Mace:EC_{50} = 5.608 μmol. *D*: Macelignan induced adipogenesis. Oil red O staining (*upper*) and triglyceride accumulation (*lower right*) were measured after differentiation of 3T3-L1 cells in medium containing 0.1% DMSO (control), 10 μmol/l macelignan, or 10 μmol/l troglitazone for 7 days. Expression of adipocyte marker genes was measured at day 3 and day 7 using RT-PCR (*lower left*). *E*: Macelignan increased PPARγ target gene expression in 3T3-L1 adipocytes. Expression of mRNAs was estimated using quantitative real-time RT-PCR, and the results were expressed as mRNA levels relative to 0.1% DMSO (control). □, 0.1% DMSO (control); ■, Mace 10 μmol. *F*: Macelignan increased ligand-binding activity of PPARα. COS-7 cells were transfected with pFA-PPARα and pFR-Luc (UAS-Gal4-luciferase) then treated with macelignan or WY14643 at various concentrations for 24 h. ■, Wy:EC_{50} = 1.601 μmol; ▲, Mace:EC_{50} = 5.405 μmol. *G*: Macelignan induced transcriptional activity of PPARα. HepG2 hepatocytes were transfected with 3 × PPREs-tk-Luc and treated with macelignan or WY14643 for 24 h. Wy:EC_{50} = 1.973 μmol; ▲, Mace:EC_{50} = 6.208 μmol. *H*: Macelignan increased PPARα target gene expression in SK-HEP1 hepatocytes. □, 0.1% DMSO (control); ■, Mace 10 μmol. All data are expressed as the means ± SD of three independent experiments. *$P < 0.05$ macelignan treated vs. 0.1% DMSO.

PPRE)-tk-luciferase vector (Fig. 1*G*). However, macelignan showed less transactivation compared with the PPARα-specific full agonist WY14643. To further explore the PPARα agonist potential of macelignan, we examined expression of PPARα target genes in macelignan-treated SK-HEP1 cells. Macelignan increased expression of CD36, carnitine palmitoyltransferase (CPT)-1, pyruvate dehydrogenase kinase 4 (PDK4), and acyl-CoA oxidase (ACO) (Fig. 1*H*). Taken together, these results show that macelignan is a natural dual PPARα/γ agonist with relatively lower, but significant, PPARα/γ dual agonist activity.

Effects of macelignan on metabolic disorders in obese diabetic mice. To examine the in vivo metabolic effects of macelignan on diabetes, C57BL/KsJ-*db/db* mice were treated orally every day for 2 weeks (from 10 to 12 weeks of age) with macelignan (10 or 25 mg/kg). Treatment effects were compared with troglitazone 10 mg/kg. While food intake was not different between macelignan-treated and troglitazone-treated or untreated *db/db* mice (Fig. 2*A*), macelignan-treated mice decreased in body weight slightly; meanwhile, the body weights of the troglitazone-treated and untreated *db/db* mice increased (Fig. 2*B*). Interestingly, gross inspection of macelignan-treated mice revealed a clear reduction in white adipose tissue mass compared with troglitazone-treated or untreated *db/db* mice (Fig. 2*C*, *left*) and increase in small size of adipocytes than in untreated *db/db* mice (Fig. 2*C*, *right*). Nonfasting blood glucose and insulin levels were signifi-

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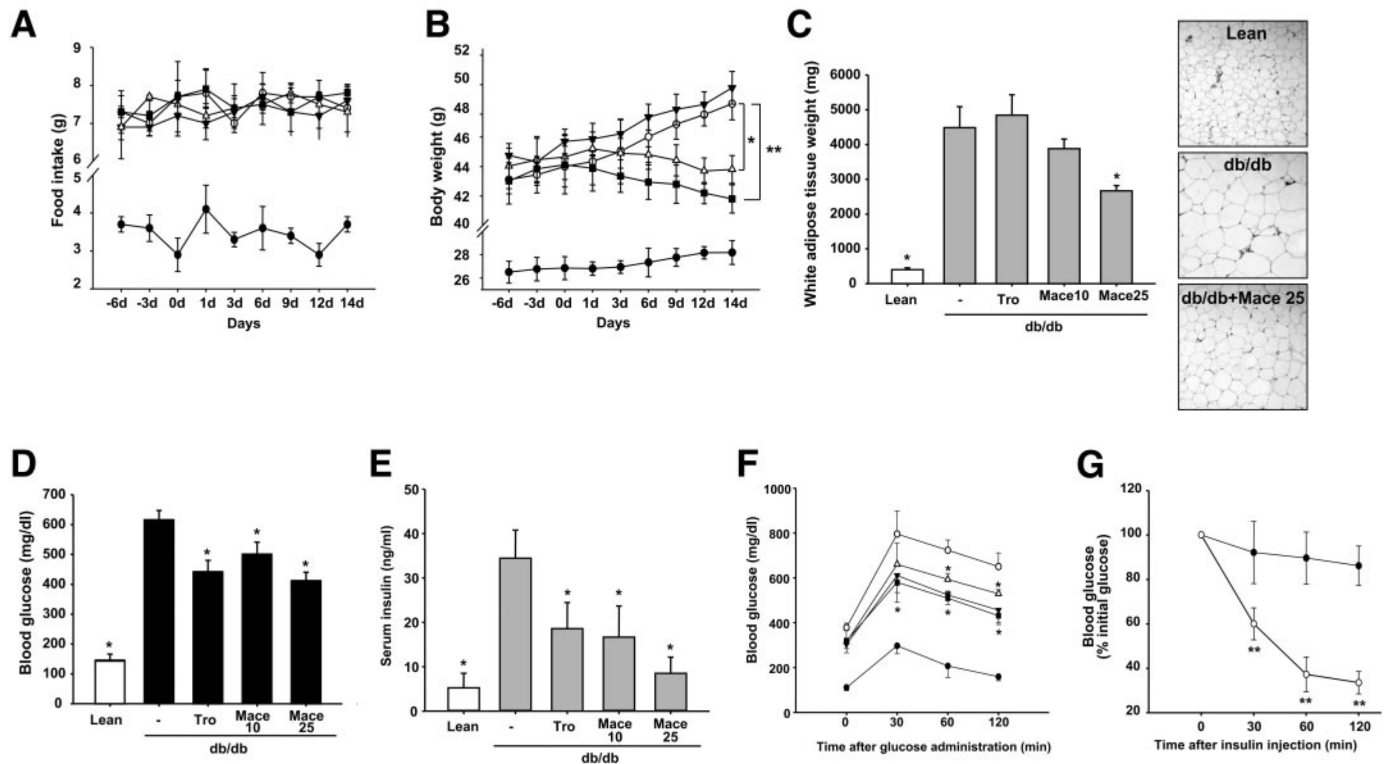

FIG. 2. Macelignan ameliorates glucose tolerance/insulin resistance in *db/db* mice. Mice were treated for 14 days at different doses of macelignan or troglitazone. *A*: Effect of macelignan on food intake. ■, Lean; □, *db/db*; ▼, *db/db* + Tro; △, *db/db* + Mace 10; ■, *db/db* + Mace 25. *B*: Effect of macelignan on body weight. ■, Lean; □, *db/db*; ▼, *db/db* + Tro; △, *db/db* + Mace 10; ■, *db/db* + Mace 25. *C*: Effect of macelignan on weight (*left panel*) and morphology of white adipose tissue (*right panel*). Histological examination with hematoxylin-eosin staining. Total magnification was ×100. *D*: Effect of macelignan on nonfasting blood glucose levels. *E*: Effect of macelignan on nonfasting insulin levels. *F*: Effect of macelignan on glucose tolerance in *db/db* mice. ■, Lean; □, *db/db*; ▼, *db/db* + Tro; △, *db/db* + Mace 10; ■, *db/db* + Mace 25. *G*: Effect of macelignan on insulin tolerance in *db/db* mice. ●, *db/db*; ○, *db/db* + Mace 10. Data represent are shown as the mean ± SD of seven different animals. *P < 0.05 as compared with untreated *db/db* mice. **P < 0.01 as compared with untreated *db/db* mice. Tro, troglitazone 10 mg · kg^{-1} · day^{-1}; Mace 10, macelignan 10 mg · kg^{-1} · day^{-1}; Mace 25, macelignan 25 mg · kg^{-1} · day^{-1}.

cantly lower in mice treated with macelignan than in untreated *db/db* mice and nearly equal to those found in troglitazone-treated mice (Fig. 2*D* and *E*). To assess glucose homeostasis and insulin sensitivity in *db/db* mice treated with macelignan, glucose tolerance and insulin tolerance tests were performed in 12-week-old mice. As shown in Fig. 2*F* and *G*, 10 and 25 mg/kg macelignan improved blood glucose levels and glucose tolerance in *db/db* mice (Fig. 2*F*). The insulin tolerance test also showed that reduction in blood glucose levels in response to insulin was much greater in mice treated with 10 mg/kg macelignan than in untreated *db/db* mice (Fig. 2*G*). When the effects of macelignan on insulin signaling were investigated in insulin target tissues, macelignan suppressed basal serine^{307} phosphorylated levels of IRS-1 in the skeletal muscle of *db/db* mice compared with untreated *db/db* mice, whereas tyrosine phosphorylation increased in macelignan-treated *db/db* mice (online appendix Fig. 1*A* [available at http://dx.doi.org/10.2337/db07-0972]). Similar results were observed in both liver and white adipose tissue of macelignan-treated *db/db* mice (online appendix Fig. 1*B* and *C*).

Next, the effects of macelignan on serum triglycerides and FFA levels and triglyceride levels in the liver and skeletal muscle were investigated. Macelignan decreased serum triglycerides and FFA levels and reduced triglycerides in both tissues in a dose-dependent manner compared with untreated *db/db* mice, which was correlated with an increase of β-oxidation (Table 1). Furthermore, macelignan increased serum HDL cholesterol. Together, these results demonstrate that macelignan improves plasma glucose and lipid profiles in *db/db* mice.

Effects of macelignan on the expression of metabolic genes and adipocytokines. Next, to explore the molecular mechanisms by which macelignan improved insulin resistance in *db/db* mice, we first examined expression of PPARα and -γ target genes involved in metabolic processes occurring in white adipose tissue and liver, respectively. The expression of CD36 and LPL genes involved in fatty acid entry as well as acyl-CoA synthetase and glycerol kinase genes involved in FFA recycling increased in the white adipose tissue of macelignan-treated *db/db* mice compared with untreated *db/db* mice (Fig. 3*A*). The expression of PPARα target genes involved in fatty acid oxidation, including CPT-1, LPL, ACO, and cytochrome P-450 4A (CYP4A) genes, also increased in the liver of macelignan-treated mice (Fig. 3*B*). Furthermore, we measured expression of genes associated with energy expenditure such as uncoupling proteins (UCPs). As shown in Fig. 3*C*, macelignan administration caused a 1.8-fold induction of UCP3 expression in the skeletal muscle of *db/db* mice. The expression and serum levels of adipocytokines were investigated as PPARγ agonists regulate expression of adipocytokine genes implicated in glucose homeostasis and insulin sensitivity. As shown in Fig. 3*D*, macelignan greatly increased adiponectin expression in white adipose tissue as well as serum adiponectin levels in *db/db* mice, whereas expression and serum levels of TNF-α and IL-6

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TABLE 1
Maceligan decreases serum triglylcerides, FFAs, and tissue triglycerides in *db/db* mice

	Serum						
db/db mice	Triglycerides (mg/dl)	FFAs (mEq/l)	HDL cholesterol (ng/ml)	Triglycerides in liver (mg/g)	Triglycerides in muscle (mg/g)	β-Oxidation in liver (% of *db/db* untreated mice)	β-Oxidation in muscle (% of *db/db* untreated mice)
Lean mice	72.57 ± 36.36†	0.76 ± 0.27*	35.74 ± 6.41*	1.95 ± 0.79*	11.05 ± 5.12*	298.79 ± 33.94*	452.31 ± 38.20*
Untreated	279.29 ± 67.89	1.79 ± 0.34	23.07 ± 8.97	10.32 ± 1.72	27.62 ± 2.44	100.00 ± 22.42	100.00 ± 19.63
Troglitazone (10 mg/kg)	112.57 ± 57.47*	1.42 ± 0.27	43.85 ± 6.34*	8.87 ± 3.57	28.15 ± 4.98	113.41 ± 33.16	102.62 ± 42.32
Mace (10 mg/kg)	91.71 ± 44.04†	1.06 ± 0.31*	42.17 ± 7.29*	6.76 ± 2.75	22.80 ± 5.76	152.53 ± 20.74*	132.47 ± 28.38
Mace (25 mg/kg)	84.00 ± 31.24†	1.04 ± 0.15*	46.58 ± 3.89*	6.30 ± 1.59*	20.24 ± 3.82*	184.27 ± 27.95*	159.58 ± 31.35*

Data are means ± SD. *db/db* mice (10-week-old females, seven mice per group) were treated with troglitazone (10 mg · kg^{-1} · day^{-1}) or macelignan (10 or 25 mg · kg^{-1} · day^{-1}) for 2 weeks. **P* < 0.05 compared with untreated *db/db* mice group. †*P* < 0.01 compared with untreated *db/db* mice group.

were reduced compared with untreated *db/db* mice (Fig. 3*E*). These results suggest that macelignan might improve insulin resistance by regulating fatty acid metabolism and adipocytokines expression in *db/db* mice.

Effects of macelignan on inflammatory processes and AMP-activated protein kinase activation. As inflammatory processes play potential roles in the pathogenesis of insulin resistance, we investigated whether macelignan possesses antiinflammatory effects. To this end, we examined the expression profile of proinflammatory cytokines in the liver of macelignan-treated *db/db* mice. While untreated *db/db* mice showed elevated levels of TNF-α, IL-6, IL-1β, and C-reactive protein compared with normal lean mice, treatment with macelignan reduced the expression of these cytokines significantly (Fig. 4*A*). Since the IκB kinase β/nuclear factor (NF) κB pathway drives the production of numerous proinflammatory cytokines and is also considered a molecular mediator of insulin resistance, we assessed the effects of macelignan on IκB kinase β/NFκB signaling in the liver. As shown in Fig. 4*B*, macelignan suppressed elevated phosphorylation of inhibitor of κBα (IκBα), a bound inhibitor of NFκB, in the livers

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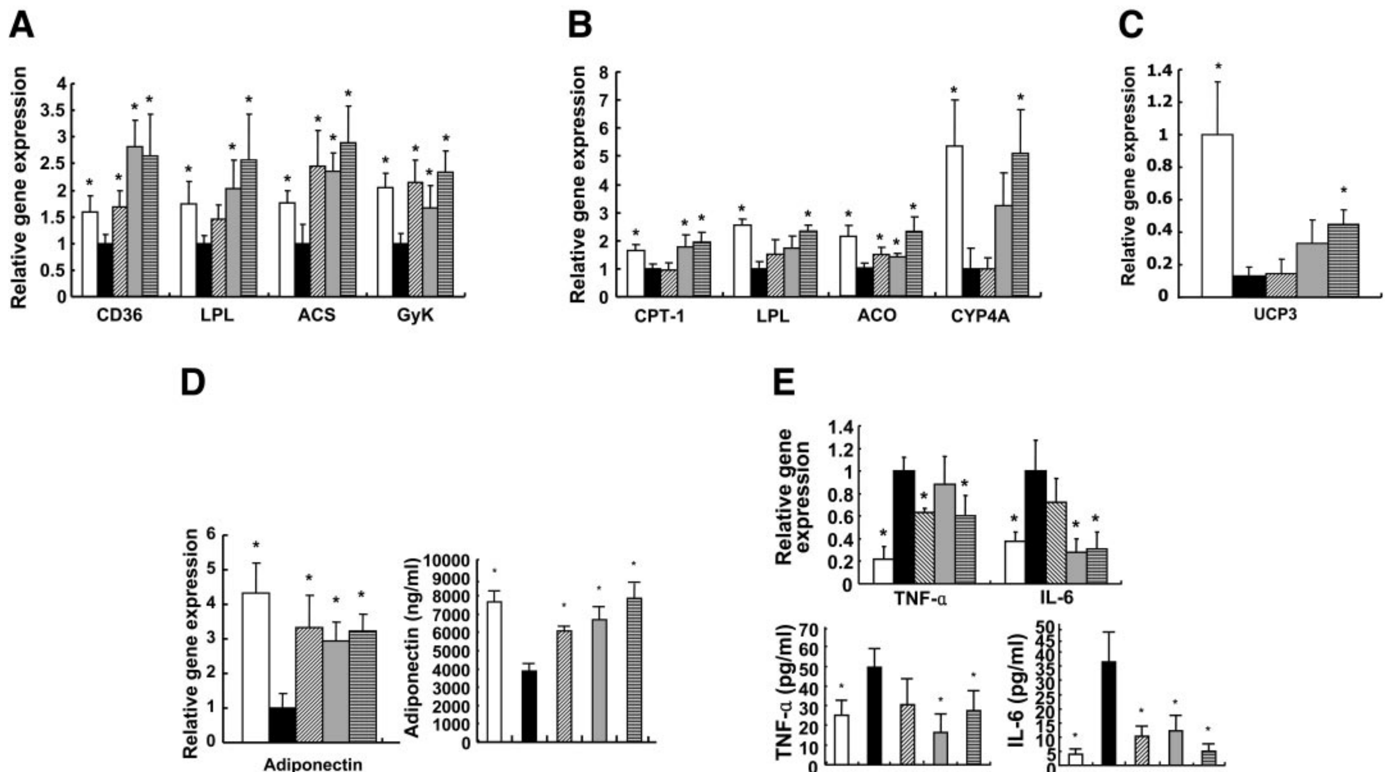

FIG. 3. Macelignan increases expression of PPARγ and -α target genes in white adipose tissue and liver of *db/db* mice and regulates adipocytokines expression and serum levels. After administration of macelignan for 14 days, total RNAs were isolated from white adipose tissue and liver and subjected to quantitative real-time RT-PCR. *A*: Macelignan increased expression of PPARγ target genes in white adipose tissue of *db/db* mice. *B*: Macelignan increased expression of PPARα target genes in liver of *db/db* mice. *C*: Macelignan increased expression of UCP3 gene in skeletal muscle of *db/db* mice. *D*: Macelignan increased adiponectin expression (*left*) and serum level (*right*). *E*: Macelignan decreased expression (*upper*) and serum levels (*lower*) of TNF-α and IL-6. mRNA expression was expressed as values relative to untreated *db/db* mice. Data are shown as the mean ± SD of seven different animals. □, Lean; ■, *db/db*; ▨, *db/db* + Tro; ▒, *db/db* + Mace 10; ▤, *db/db* + Mace 25. *P < 0.05 compared with untreated *db/db* mice.

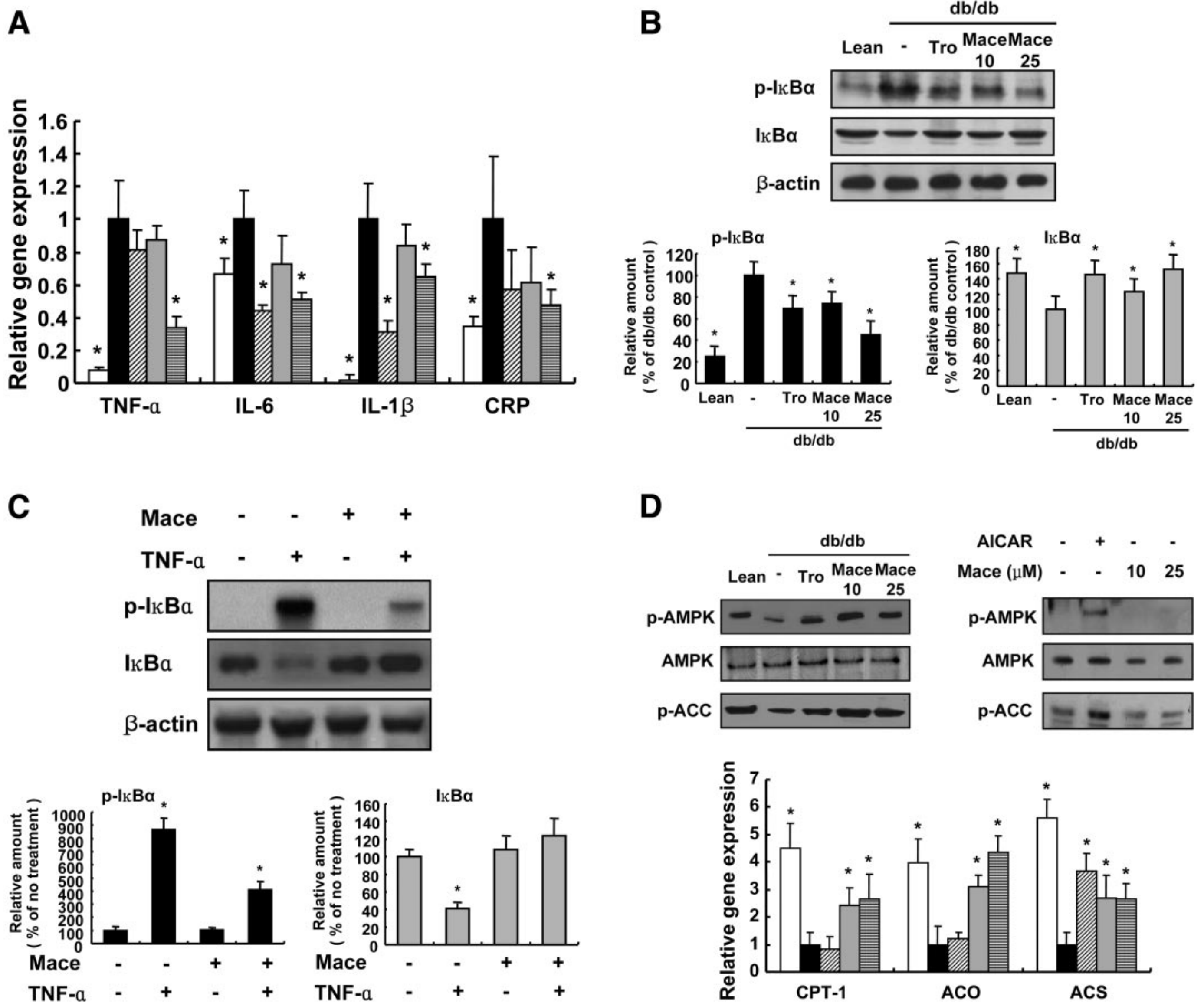

FIG. 4. Macelignan inhibits inflammatory processes in the liver and activates AMPK in the skeletal muscle of *db/db* mice. *A*: Macelignan decreased expression of inflammatory cytokines genes in the liver of *db/db* mice. mRNA expression was estimated using quantitative real-time RT-PCR. □, Lean; ■, *db/db*; ▨, *db/db* + Tro; ▩, *db/db* + Mace 10; ▤, *db/db* + Mace 25. *B*: Macelignan decreased IκBα phospholyration in the liver of *db/db* mice. Total proteins obtained from livers of *db/db* mice after 12 h fasting were subjected to Western blot with IκBα or phosphorylated IκBα antibody. *C*: Macelignan decreased IκBα phospholyration induced by TNF-α in HepG2 hepatocytes. HepG2 cells was preincubated with macelignan (10 μmol) for 24 h and then treated with TNF-α (10 ng/ml) for 20 min. Phosphorylated IκBα and IκBα were measured by Western blot analysis. *D*: Macelignan increased phosphorylation of AMPK (*upper left*) and expression of fatty acid oxidation genes in skeletal muscle (*lower*) of *db/db* mice. Macelignan did not activate AMPK directly in differentiated C2C12 cells (*upper right*). C2C12 cells were treated with aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (1 mmol/l) or macelignan (10 μmol/l) for 24 h. Phosphorylated AMPK was examined by Western blot analysis, and mRNA expression was estimated using quantitative real-time RT-PCR. Data are shown as the means ± SD of seven different animals or three independent experiments. □, Lean; ■, *db/db*; ▨, *db/db* + Tro; ▩, *db/db* + Mace 10; ▤, *db/db* + Mace 25. *$P < 0.05$ compared with untreated *db/db* mice or HepG2 cells.

of *db/db* mice, which subsequently increased IκBα levels. To further examine the antiinflammatory effects of macelignan, we evaluated the inhibitory effects of macelignan on IKKβ/NFκB signaling in HepG2 cells treated with TNF-α. While TNF-α treatment induced IκBα phosphorylation and reduced IκB levels, treatment with macelignan inhibited TNF-α–induced IκBα phosphorylation and thus increased IκBα levels (Fig. 4*C*). When insulin signaling was examined in TNF-α–treated HepG2 cells, TNF-α increased serine phosphorylation of IRS-1, but the serine phosphorylation was significantly reduced by macelignan treatment (online appendix Fig. 2*A*). Furthermore, TNF-α decreased tyrosine phosphorylation of IRS-1 stimulated by insulin, whereas macelignan treatment recovered the tyrosine phosphorylation (online appendix Fig. 2*B*). These results were consistent with threonine phosphorylation of of Akt, indicating that macelignan exerts anti-inflammatory effects, which may be involved in improving insulin resistance.

Next, to determine whether macelignan mediates AMP-activated protein kinase (AMPK) activation, we measured AMPK phosphorylation and expression of fatty acid oxidation genes in the skeletal muscle of *db/db* mice. As shown in Fig. 4*D*, AMPK phosphorylation increased significantly in macelignan-treated mice compared with untreated *db/db* mice, and phosphorylated acetyl CoA carboxylase, a

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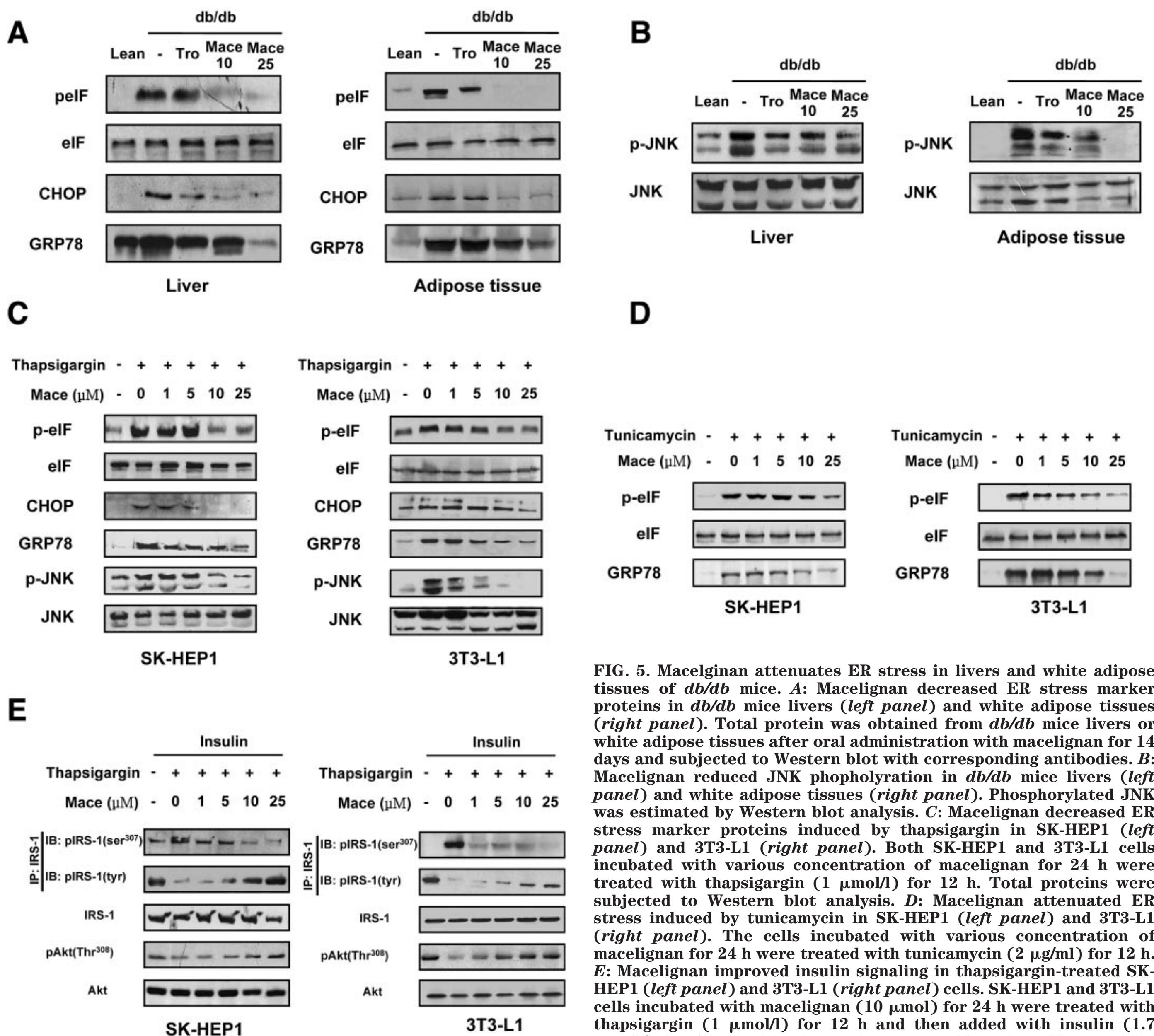

FIG. 5. Macelignan attenuates ER stress in livers and white adipose tissues of *db/db* mice. *A*: Macelignan decreased ER stress marker proteins in *db/db* mice livers (*left panel*) and white adipose tissues (*right panel*). Total protein was obtained from *db/db* mice livers or white adipose tissues after oral administration with macelignan for 14 days and subjected to Western blot with corresponding antibodies. *B*: Macelignan reduced JNK phopholyration in *db/db* mice livers (*left panel*) and white adipose tissues (*right panel*). Phosphorylated JNK was estimated by Western blot analysis. *C*: Macelignan decreased ER stress marker proteins induced by thapsigargin in SK-HEP1 (*left panel*) and 3T3-L1 (*right panel*). Both SK-HEP1 and 3T3-L1 cells incubated with various concentration of macelignan for 24 h were treated with thapsigargin (1 μmol/l) for 12 h. Total proteins were subjected to Western blot analysis. *D*: Macelignan attenuated ER stress induced by tunicamycin in SK-HEP1 (*left panel*) and 3T3-L1 (*right panel*). The cells incubated with various concentration of macelignan for 24 h were treated with tunicamycin (2 μg/ml) for 12 h. *E*: Macelignan improved insulin signaling in thapsigargin-treated SK-HEP1 (*left panel*) and 3T3-L1 (*right panel*) cells. SK-HEP1 and 3T3-L1 cells incubated with macelignan (10 μmol) for 24 h were treated with thapsigargin (1 μmol/l) for 12 h and then added with insulin (1.7 μmol/l) for 15 min. Total proteins were subjected to Western blot analysis. Total proteins were immunoprecipitated with IRS-1 antibody, and immunoprecipitates were subjected to Western blot with serine (307) phosphorylated IRS-1 antibody or phosphotyrosine antibody. Threonine (308) phosphorylated Akt was estimated by Western blot analysis. Blots are representative from seven different animals or three independent experiments.

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downstream signal of AMPK, also increased after macelignan treatment (Fig. 4*D*, *upper left*). Consistent with AMPK activation, expression of fatty acid oxidation genes, including CPT-1, ACO, and acyl-CoA synthetase, increased in macelignan-treated *db/db* mice (Fig. 4*D*, *lower*). To determine whether AMPK activation is a direct effect of macelignan treatment, we examined AMPK phosphorylation in macelignan-incubated C2C12 cells. While aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, an AMPK activator, increased AMPK phosphorylation in C2C12 cells, macelignan treatment did not activate AMPK in C2C12 cells, which is similar to results for acetyl CoA carboxylase phosphorylation (Fig. 4*D*, *upper right*), suggesting that macelignan does not activate AMPK directly.

Effects of macelignan on ER stress. It has been recently suggested that ER stress plays a central role in the development of insulin resistance and diabetes by impairing insulin signaling through c-Jun NH_2-terminal kinase (JNK) activation. To further characterize the molecular mechanisms underlying macelignan's antidiabetes properties, we investigated whether macelignan alleviates ER stress in the liver and white adipose tissue of *db/db* mice. As shown in Fig. 5*A*, markers of ER stress, such as phosphorylated eukaryotic initiation factor, CCAAT/enhancer-binding protein homologous protein, and glucose-regulated protein (GRP)78 were increased in *db/db* mice compared with lean mice, but macelignan administration reduced ER stress indicators in both the liver and white adipose tissue of *db/db* mice. Treatment with troglitazone did not significantly affect ER stress, although GRP78 was slightly decreased in the liver of troglitazone-treated *db/db* mice. Consistent with the results on ER stress indicators, JNK phosphorylation was also significantly suppressed in both the liver and white adipose tissue upon macelignan

treatment (Fig. 5*B*). To confirm that the observed protective effects against ER stress were the direct result of macelignan, we examined ER stress indicators and insulin signaling in thapsigargin-incubated SK-HEP1 cells and 3T3-L1 cells. While thapsigargin incubation increased ER stress indicators, phosphorylated eukaryotic initiation factor, CCAAT/enhancer-binding protein homologous protein, GRP78, and JNK, treatment with macelignan suppressed thapsigargin-induced effects in both cell lines in a dose-dependent manner (Fig. 5*C*). Similar results were also found in tunicamycin-incubated cells (Fig. 5*D*). Macelignan treatment also suppressed serine phosphorylation of IRS-1 induced by thapsigargin and thus improved tyrosin phosphorylation of IRS-1 and threonine phosphorylation of Akt (Fig. 5*E*). In contrast, treatment with troglitazone did not alleviate ER stress in thapsigargin-incubated SK-HEP1 cells and 3T3-L1 cells (data not shown). We also investigated the effects of macelignan on thapsigargin-induced apoptosis of pancreatic β-cells, MIN6N8. As shown in online appendix Fig. 3, macelignan treatment decreased the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling–positive MIN6N8 cells induced by thapsigargin (online appendix Fig. 3, *left*) and increased viable cells (online appendix Fig. 3, *right upper panel*) in a dose-dependent manner, suggesting that macelignan can protect the cells from apoptosis induced by ER stress. Insulin secretion reduced by thapsigargin treatment was rescovered by macelignan, not by troglitazone (online appendix Fig. 3, *right lower panel*).

DISCUSSION

A number of research groups have conducted extensive investigations in an effort to develop efficacious dual PPAR α/γ agonists and develop potential agents such as ragaglitazar, tesaglitazar, muraglitazar, and chromane-2-carboxylic acids derivatives (15–18). However, the use of these agents has been discontinued because of serious side effects, including carcinogenesis (19).

We have characterized the effects of macelignan, a natural PPAR α/γ dual agonist isolated from *Myristica fragrans*. Previously, macelignan was found to possess therapeutic potential against neurodegenerative diseases as it was known to display antioxidant and antiinflammatory activities (20). Here, we have shown that macelignan functions as a PPAR α/γ dual agonist, but the potency was less than WY14643, a full PPARα agonist, and troglitazone, a PPARγ agonist, in in vitro transactivation and transient transfection. Moreover, macelignan activity was a more potent agonist for PPARα relative to PPARγ. When administered to *db/db* mice, macelignan significantly ameliorated insulin resistance and exerted beneficial effects on fatty acid and glucose metabolism in *db/db* mice similar to troglitazone. These effects suggest that macelignan could provide a suitable therapeutic approach to the treatment of type 2 diabetes, metabolic syndrome, and associated vascular disorders.

The molecular mechanisms of macelignan contributing to improved insulin sensitivity and hyperlipidemia were investigated. Macelignan upregulated adipocyte genes involved in FFA entry, including LPL and aP2, as well as genes involved in FFA recycling within the adipocyte, such as glycerol kinase, which might cause secondary effects that reduce exposure to fatty acids in the skeletal muscles and liver. Macelignan increased production of insulin-sensitizing adiponectin and reduced insulin resistance–mediating adipocytokines such as TNF-α and IL-6. These effects may all contribute to improved insulin resistance in *db/db* mice. Macelignan treatment was also found to increase expression of LPL, CPT-1, ACO, and CYP4A, involved in fatty acid entry, fatty acid oxidation, and hydroxylation of fatty acid in *db/db* mice livers and expression of UCP3 involved in energy expenditure in skeletal muscle. Thus, these effects may lead to reduced synthesis and secretion of triglycerides and thereby decrease liberation of fatty acids, resulting in a slight reduction in body weight and white adipose tissue weight in macelignan-treated *db/db* mice compared with troglitazone-treated or untreated mice. Based on these results, macelignan treatment can reverse weight gain typically observed with PPARγ agonists.

A close connection between insulin resistance and inflammatory signaling pathways has been identified. The inflammatory cytokine and IKKβ attenuates insulin signaling through serine phosphorylation of IRS-1. High doses of salicylates, which block IKKβ activity, ameliorate hyperglycemia and insulin resistance in diabetes and obesity (21). Our results show that macelignan effectively suppressed expression of inflammatory genes including TNF-α, IL-6, IL-1β, and C-reactive protein normally induced in both the liver and white adipose tissue of *db/db* mice and significantly inhibited IKKβ activity in *db/db* mice as well as in TNF-α–treated HepG2 cells, indicating that macelignan blocked activation of NFκB pathways. Consistent with inhibition of NFκB activation, insulin signaling was ameliorated after macelignan treatment in TNF-α–incubated HepG2 cells. These results demonstrate that macelignan can improve insulin sensitivity through inhibition of NFκB activation and suppression of inflammatory genes. Furthermore, activation of AMPK enhances insulin sensitivity through increased glucose uptake and lipid oxidation in skeletal muscle and inhibition of glucose and lipid synthesis in the liver (22). In the current study, macelignan was shown to activate AMPK in the skeletal muscle of *db/db* mice; however, since macelignan treatment did not activate AMPK in C2C12 cells, the AMPK activation observed in skeletal muscle may not be a direct effect of macelignan but rather a secondary effect due to elevated adiponectin levels.

Recently, ER stress has received growing attention as it has been linked to type 2 diabetes pathogenesis through triggering JNK activity and inhibition of insulin signaling (23,24). Therefore, agents that alleviate ER stress may act as potent antidiabetes agents with potential application in the treatment of type 2 diabetes. Previous reports have shown that administration of active chemical chaperones that modulate ER stress by increasing folding capacity in obese and diabetic mice results in normalization of hyperglycemia, restoration of systemic insulin sensitivity, and enhancement of insulin activity in liver, skeletal muscle, and white adipose tissues (25). Therefore, we also investigated whether macelignan could alleviate ER stress in the liver and white adipose tissue of *db/db* mice. Whereas indicators of ER stress and phosphorylation of JNK were increased in *db/db* mice, treatment with macelignan markedly reduced ER stress indicators and JNK phosphorylation. In thapsigargin or tunicamycin-incubated SK-HEP1 cells and 3T3-L1 cells, macelignan treatment also suppressed ER stress indicators, which demonstrates the direct protective effects of macelignan against ER stress. Consistent with these results, macelignan enhanced both

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IRS-1 tyrosine phosphorylation and Akt threonine phosphorylation reduced by thapsigargin treatment. Furthermore, maceligan protected pancreatic β-cells from the apoptosis induced by ER stress. All these results indicate that both insulin signaling and insulin secretion can be recovered with macelignan through reduced ER stress in *db/db* mice.

Given these results, macelignan presents many therapeutic advantages for the treatment of type 2 diabetes and associated diseases. As macelignan is a purified component of an edible plant, it can be suitable to be a lead compound toward promising antidiabetes agent. Even though in vitro assays showed that macelignan had only partial agonist activities on PPARα and -γ compared with full agonists, macelignan achieved the same degree of efficacy as troglitazone in vivo. Furthermore, macelignan showed more efficient activity on PPARα relative to PPARγ, which can alleviate side effects, such as increased body weight and edema associated with PPARγ agonists. Also, macelignan displayed more beneficial effects on triglycerides, FFAs, and HDL cholesterol than troglitazone. This combined with macelignan's anti-inflammatory activity, suggests that it could be a good therapeutic agent for atherosclerosis. Most importantly, in contrast to troglitazone, macelignan also has the ability to alleviate ER stress and therefore can be utilized in diseases induced by ER stress, including neurodegenerative disorders. Our results indicate that macelignan can act as a potent antidiabetes agent with potential application in treating type 2 diabetes.

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