A PPAR Pan Agonist, MHY2013 Alleviates Age-Related Hepatic Lipid Accumulation by Promoting Fatty Acid Oxidation and Suppressing Inflammation

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Nonalcoholic fatty liver disease (NAFLD) is frequently observed in obese and aged individuals. Peroxisome proliferator-activated receptors (PPARs) play a role in regulating hepatic lipid accumulation, a hallmark of NAFLD development. A PPAR pan agonist, 2-(4-(5,6-methylenedioxybenzo[d]thiazol-2-yl)-2-methylphenoxy)-2-methylpropanoic acid (MHY2013) has been shown to prevent fatty liver formation and insulin resistance in obese mice (db/db) model. However, the beneficial effects of MHY2013 in aged model remain unknown. In this study, we investigated whether MHY2013 alleviates hepatic lipid accumulation in aged Sprague–Dawley (SD) rats. We confirmed that MHY2013 increased the activities of three PPAR subtypes in HepG2 cells using luciferase assay. When administered orally in aged SD rats, MHY2013 markedly decreased the hepatic triglyceride levels without changes in body weight. Regarding underlying mechanisms, MHY2013 increased the mRNA levels of lipid oxidation-related genes, including carnitine palmityltransferase 1 (CPT1) and peroxisomal acyl-CoA oxidase 1 (ACOX1), without apparent change in the mRNA expression of lipogenesis-related genes. Furthermore, MHY2013 significantly increased systemic fibroblast growth factor 21 (FGF21) and adiponectin levels and suppressed inflammatory mRNA expression in the liver. In conclusion, MHY2013 alleviated age-related hepatic lipid accumulation, in part by upregulating β-oxidation signaling and suppressing inflammation in the liver. Therefore, MHY2013 is a potential pharmaceutical agent for treating age-related hepatic lipid accumulation.

Key words aging; nonalcoholic fatty liver disease; MHY2013; peroxisome proliferator-activated receptor (PPAR) pan agonist; lipid oxidation

Aging of vast majority of population is very recent phenomenon that has emerged as a direct consequence of the extension of lifespan. The aging process cause hepatic functional and structural impairments leading to metabolic risks.1 Notably, aging is closely associated with the occurrence of nonalcoholic fatty liver disease (NAFLD), which is characterized by a physiological increase in lipid accumulation in the liver. Lipid accumulation in the liver may impair its normal function by promoting lipotoxicity.2 Based on a previous report, lipids may accumulate in the liver as a result of multiple abnormalities in hepatic lipid metabolism including enhanced fat uptake and lipid synthesis and suppressed lipolysis and lipid oxidation.3 In consistent with this suggestion, some studies have reported that lipid oxidation signaling pathway was downregulated in the liver during aging and it is accompanied with reduced expression and activation of peroxisome proliferator-activated receptors (PPARs).4 PPARs play a pivotal role in regulating various cellular processes related to energy metabolism, including gluconeogenesis, lipid synthesis, lipid uptake, and lipid breakdown.6 PPARα is mainly expressed in the liver, and it induces β-oxidation and energy expenditure.7 In addition, it directly upregulates the expression of the hepatokine, fibroblast growth factor 21 (FGF21), which is considered a promising intervention target for metabolic diseases owing to its insulin-sensitizing and thermogenesis-inducing effects.8 However, PPARγ is predominantly expressed in the adipose tissue, where it regulates adipocyte differentiation and insulin sensitivity.9 PPARγ is a key regulator of adiponectin, a well-known insulin sensitizer. Although biological roles of PPAR δ need to be clarified, a variety of studies have demonstrated that it is associated with glucose and lipid metabolism. Thus, the PPARs are among the most remarkable targets for treating metabolic diseases including NAFLD, type 2 diabetes, etc.10

Our previous study has revealed that 2-(4-(5,6-methylenedioxybenzo[d]thiazol-2-yl)-2-methylphenoxy)-2-methylpropanoic acid (MHY2013) is a potent PPAR pan agonist and prevent obesity-induced metabolic syndromes such as fatty liver, dyslipidemia and insulin resistance.11 As underlying mechanisms, we have suggested the elevation of hormones, such as FGF21 and adiponectin, white adipose tissue (WAT) browning markers, and fatty acid oxidation in the liver and skeletal muscles may synergistically contribute to the beneficial effects of MHY2013. In this study, we investigated whether the PPAR pan agonist MHY2013 attenuates fatty liver formation in aged Sprague–Dawley (SD) rats. Our data showed that MHY2013 can activate the three PPAR subtypes, leading to the alleviation of hepatic steatosis with induced lipid oxidation and suppressed inflammation in aged rats. Our study provides the molecular rationale for MHY2013 as a new marker of age-related steatosis.
pharmaceutical compound that can intervene with age-related metabolic syndrome.

MATERIALS AND METHODS

Animal Experiments Male, SD rats (5 and 19 months) were purchased from Samtako Bio Korea (Osan, Republic of Korea) and acclimated to the animal care facility for seven days before the experiment. The animals were housed in an air-conditioned environment, under a 12h light/dark cycle, and given ad libitum access to a standard rodent chow (Samtako) diet and water. The animal study was approved by the Institutional Animal Care Committee of Pusan National University (2, Busandaehak-ro 63beon-gil, Geumjeong-gu, Busan, Republic of Korea) and performed in accordance with the guidelines for animal experiments issued by the Pusan National University. Vehicle (water) and MHY2013 (1 and 5 mg/kg/d) were injected by oral gavage for four weeks. Food intakes and body weights were monitored every 5–8 d. The animals were euthanized, and the serum and tissues were collected.

Cell Culture System HepG2, a human liver cancer cell line, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Hyclone, Inc., Logan, UT, U.S.A.) at 37°C in a 5% CO₂ atmosphere.

 Luciferase Assay For peroxisome proliferator response element (PPRE)-driven luciferase assay, HepG2 cells were seeded at a density of 1.5×10⁴ cells/well in a 96-well cell culture plate. The PPRE-X3-TK-LUC plasmid (0.1 µg; kindly gifted by Dr. Christopher K. Glass, University of California, San Diego, CA, U.S.A.) and 0.01 µg PPARα, PPARβ/δ, and PPARγ expression vectors (kindly gifted by Dr. Han Geuk Seo, Konkuk University, Seoul, South Korea) were transfected into the cells using 0.11 µL Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. After incubation for 24 h, the cells were treated with the vehicle [dimethyl sulfoxide (DMSO)], 10 µM MHY2013, WY14643 (a known PPARα agonist), and rosiglitazone (a known PPARγ agonist) for 6 h. The luciferase activity was measured using the One-Glo Luciferase Assay System (Promega, Madison, WI, U.S.A.) and a luminometer (Berthold Technologies GmbH & Co., Bad Wildbad, Germany).

 Isolation of RNA and Quantitative Real-Time PCR (qRT-PCR) The RNA from tissue and cell samples was purified using the RiboEx Total RNA (GeneAll Biotechnology, Seoul, South Korea) according to the manufacturer’s instruction. Total RNA (2.0 µg) treated with ribonuclease (RNase)-free deoxyribonuclease (DNase) was reverse-transcribed with a Hyperscript™ One-Step RT-PCR (GeneAll Biotechnology, Seoul, South Korea). qRT-PCR was performed using the SensiFAST™ SYBR® No-ROX kit (BIOLINE, U.K.) and a CFX Connect System (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). The primer sequences are shown in Supplementary Table 1.

 Serum Biochemical Analysis Serum glucose, cholesterol, triglyceride (TG), non-esterified fatty acid (NEFA), and creatinine were analyzed using kits from Bioassay Systems (Hayward, CA, U.S.A.). To measure the serum adiponectin level, the Mouse Adiponectin enzyme-linked immunosorbent assay (ELISA) Kit (CircuLex Co., Bangkok, Thailand) was used. The serum FGF21 level was evaluated using the Mouse/Rat FGF-21 Quantikine ELISA Kit (R&D Systems, Minneapolis, U.S.A.). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a kit from Stabio (Boerne, TX, U.S.A.).

 Liver TG Measurements The liver samples were homogenized in ice-cold phosphate-buffered saline (PBS; Gibco, Grand Island, NY, U.S.A.). The TGs were extracted with methanol–chloroform (1:2), dried, and suspended in 5% bovine serum albumin. The TG level was evaluated using a kit from Bioassay Systems (Hayward, CA, U.S.A.).

 Statistical Analysis All in vivo experimental data are expressed as the mean±standard deviation (S.D.). In vitro data are expressed as the mean±standard error (S.E.). Statistical significance of the differences between the groups was determined by one-way ANOVA, followed by a Dunnett’s test. An associated probability (p value)<0.05 was considered significant.
RESULTS

**MHY2013 Activates Three PPAR Subtypes** To confirm that MHY2013 is a powerful PPAR pan agonist, PPRE-luciferase assays for the three PPAR subtypes were performed in HepG2 cells. Among the newly synthesized six derivatives based on the structure of the PPAR pan agonist bezafibrate (supplementary Table 1), MHY2013 had the highest luciferase activity for all PPAR subtypes, and its effect on each PPAR subtype was higher than that of each selective PPAR agonist (PPARα: WY14643, PPARβ/δ: GW501516, and PPARγ: rosiglitazone; Figs. 1A–C).

**MHY2013 Improves Serum Glucose and Lipid Profiles in Aged Rats** Severe alterations in blood nutrient and metabolite profiles are frequently observed during the aging process, and these alterations are closely related to metabolic syndrome. Our previous study reported that MHY2013 attenuates metabolic syndrome, including fatty liver, dyslipidemia, and insulin resistance in db/db mice. To evaluate the beneficial effects of MHY2013 on aged models, aged SD rats (20 month) were administered with MHY2013 (1 and 5 mg/kg/d) by oral gavage for four weeks. There were no differences in the food intake and body weight between the groups (Figs. 2A, B). We first examined the effect of MHY2013 on blood profile associated with glucose and lipid metabolism. As expected, the serum insulin, TG, NEFA, and total cholesterol levels were much higher in the aged rats compared to those in young rats, confirming impaired glucose and lipid profiles in aged rats (Figs. 2C–G). However, MHY2013 reduced the serum levels of glucose and insulin in aged rats without affecting their body weight (Figs. 2C, D). Moreover, MHY2013 treatment significantly reduced the TG and NEFA levels in aged rats (Figs. 2E–F). These results suggest that MHY2013 improves glucose and lipid profiles, and this metabolic improvement could not be attributable to their body weight reduction.

**MHY2013 Increases Circulating FGF21 and Adiponectin Levels in Aged Rats** It has been reported that PPAR subtypes regulate the expression of endocrine hormones that
FGF21 is a hormone mainly secreted from the liver in response to PPAR α activation and from the adipose tissue by PPAR γ activation. FGF21 has been considered a promising intervention target for metabolic diseases owing to its insulin-sensitizing and thermogenesis-inducing effects. Besides FGF21, a well-known
insulin-sensitizing hormone adiponectin is expressed and secreted from the adipose tissue in response to PPARγ activation. Our previous studies suggested that MHY2013-mediated increases in systemic FGF21 and adiponectin levels contribute to the improvement of metabolic syndromes. Therefore, we checked the levels of circulating FGF21 and adiponectin in MHY2013-treated aged rats. Serum FGF21 and adiponectin levels increased in the MHY2013-treated aged rat owing to the activation of PPAR subtypes (Figs. 3A, B). These results suggest that the beneficial effects of MHY2013 are partially mediated via increase in the FGF21 and adiponectin levels.

**MHY2013 Attenuates Hepatic Lipid Accumulation through Promoting Lipid Oxidation and Suppressing Inflammation in Aged Rats**

Hepatic lipid accumulation is often observed in aged individuals and is commonly found together with other metabolic diseases, including insulin resistance and dyslipidemia. Because an increased blood TG level under fasting conditions is closely associated with uncontrolled hepatic lipid metabolism and MHY2013 decreased the blood TG level in aged rats, we examined the beneficial effect of MHY2013 on hepatic lipid metabolism. We found that MHY2013 alleviated age-related fatty liver formation as evidenced by a significant decrease in the liver weight and hepatic TG concentration (Figs. 4A, B). The serum levels of ALT were also elevated in aged rats. The administration of MHY2013 lowered ALT level by 42%, whereas AST level remained unaltered (Figs. 4C, D). These results are in agreement with those of a previous study indicating that the administration of MHY2013 ameliorated fat accumulation in the liver and liver dysfunction in db/db mice. As potential factors underlying the MHY2013-mediated decrease in hepatic TG level, we investigated lipid oxidation and synthesis-related signaling. The mRNA expression levels of fatty acid oxidation-related genes, including acyl-CoA oxidase 1 (ACOX1), carnitine palmitoyltransferase 1 (CPT1), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCSS2), and uncoupled protein 2 (UCP2), were increased in the livers of the MHY2013-treated aged rats (Fig. 4E), indicating that the upregulation of β-oxidation signaling contributes to the repression of hepatic lipid accumulation. However, MHY2013 did not affect the lipid synthesis pathway, evidenced by the unaltered mRNA expression of lipid synthesis-related genes (Fig. 4F). These results indicate that MHY2013-mediated induction of lipid oxidation may contribute to improvement in fat accumulation and liver dysfunction in aged rats.

Moreover, the expression of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and monocyte chemotactic protein 1 (MCP1), increased in the liver of aged rats and reduced in MHY2013-treated aged rat livers (Fig. 4G). As inflammatory cytokines have been considered factors responsible for the hepatic steatosis and insulin resistance, the reduction in inflammation elicited by MHY2013 treatment may contribute to the prevention of fatty liver formation.

**DISCUSSION**

In this study, we investigated whether the PPAR pan agonist MHY2013 attenuates lipid accumulation in aged SD rat livers. When injected orally in aged SD rats, MHY2013 greatly improved serum glucose and lipid profiles and prevented fatty liver formation without changes in body weight. Although the underlying mechanisms should be diverse, the improvement of fatty acid oxidation, serum FGF21 and adiponectin levels, and inflammation signaling in the liver may synergistically contribute to the beneficial effects of MHY2013 in aged rats.

The major aspects of lipid metabolism consist of catabolic processes, such as lipid oxidation, and anabolic processes, such as de novo lipogenesis. Several studies have revealed that lipid oxidation signaling is downregulated and lipogenesis signaling is upregulated during the aging process. It is well-known that PPAR is major regulator of lipid metabolism. As expected, the PPAR pan agonist, MHY2013 significantly increased the mRNA expression of lipid oxidation-related genes, including ACOX1 and CPT1 in aged rat model. On the other hand, MHY2013 did not affect lipogenesis-related genes, such as fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), and acetyl-CoA carboxylase (ACC). These results suggest that the reduced accumulation of TG in MHY2013 treated-aged rats may be due to an increase in β-oxidation-related signaling. In consistent with the aged rat model, mRNA expression levels of lipid oxidation-related genes were increased in the liver of the obese mouse model by MHY2013 treatment. Unlike aged rat model, mRNA expressions of lipogenesis-related genes such as FASN, SCD1, and ACC were also affected by MHY2013 treatment in obese mouse model. The mRNA expression levels of these genes were increased in obese mouse model, whereas MHY2013 decreased them. Therefore, the alleviation of fatty liver resulting from MHY2013 treatment in obese mouse may be due to both an increase in β-oxidation-related signaling and decreases in lipogenesis-related signaling.

The PPAR subtypes regulate the expression of metabolic hormones related to glucose and lipid metabolism. Among them, FGF21 is a hepatokine mainly expressed in the liver tissue. Many studies have revealed FGF21 as a mediator of pleiotropic actions of PPARα. Our previous study conducted in an obese mouse model revealed that MHY2013 increased FGF21 levels in the liver and serum. Similar to the findings in the obese mouse model, the serum FGF21 concentration increased in MHY2013-treated aged rats. Several recent studies reported that FGF21 could play a role in the progression of NAFLD by decreasing inflammation, cytokine levels, and oxidative stress. Moreover, it has been reported that PPARα-mediated FGF21 expression stimulates the thermogenic activation of brown adipose tissue (BAT) and WAT leading to the improvement of metabolic syndromes, such as NAFLD and insulin resistance. As observed in our previous study that MHY2013 markedly increased the expression of WAT browning markers and formation of multilocular lipid droplets in the WAT of db/db mice as well as 3T3-L1 adipocytes, we assume that MHY2013 has the potential to stimulate thermogenic activation of BAT and WAT in aged rats. Thus, we assume that MHY2013-induced PPARα–FGF21 endocrine signaling pathway may contribute to the amelioration of hepatic lipid accumulation in aged rats.

Adiponectin has been considered to play a relevant role in PPARγ agonist-mediated improvements in metabolic diseases. As expected, the circulating adiponectin levels were significantly increased in MHY2013-treated aged rats, similar to that observed in the obese mouse model. Moreover, our previous studies found that MHY2013 increased the mRNA expression of adiponectin in 3T3-L1 adipocytes, suggesting
that the compound can directly regulate adiponectin expression in adipocytes. Many studies have demonstrated that the glucose- and lipid-lowering effects of adiponectin appear to be mediated by hepatic 5’ AMP-activated protein kinase (AMPK) activation.25 Therefore, we assume that MHY2013-mediated increase in adiponectin levels may contribute to the reduction of lipid accumulation through AMPK activation in aged rat livers.

In NAFLD, the excess fat leads to hepatic inflammation, which is closely associated with hepatic insulin resistance, fibrosis, and apoptosis.24 We found that MHY2013 decreased the mRNA expression of pro-inflammatory cytokines, such as IL-6 and MCP1. Some studies have revealed that hepatic PPARα overexpression and activation decreased inflammatory gene expression via inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is a pro-inflammatory transcription factor.25–27 Thus, we assume that MHY2013 may inhibit hepatic inflammation via PPARα-mediated suppression of NF-κB signaling, as well as IL-6 and MCP1 expression. In addition, it is well reported that fatty acids and other lipid derivatives possibly produced from TG breakdown have been shown to induce hepatic inflammation and cause lipotoxicity.28 Because MHY2013 decreased the serum fatty acid and hepatic TG levels, it also appeared to suppress hepatic inflammation indirectly by PPARα activation.

In conclusion, MHY2013 alleviated lipid accumulation in the liver by increasing fatty acid oxidation and decreasing inflammation during the aging process. In addition, MHY2013-mediated increase in serum FGF21 and adiponectin levels may elicit the beneficial effects of the compound in aged rats. Therefore, MHY2013 is a potential pharmaceutical agent that may alleviate metabolic syndromes associated with aging. Our studies involving obese and aged models strongly support the concept of a pan PPAR therapeutic approach for the treatment of conditions that comprise the metabolic syndrome.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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