Supplementation of the Flavonoid Myricitrin Attenuates the Adverse Metabolic Effects of Long-Term Consumption of a High-Fat Diet in Mice

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ABSTRACT The flavonoid myricitrin exhibits various pharmacological and physiological effects. However, studies on the effects of myricitrin on obesity are limited. We hypothesized that dietary myricitrin would attenuate the adiposity and metabolic dysfunction that occur in obesity. To test this hypothesis, mice were randomly fed a high-fat diet (HFD) or HFD supplemented with myricitrin for 16 weeks. Myricitrin significantly reduced white adipose tissue (WAT) mass, adipocyte size, and plasma leptin levels, and also attenuated dyslipidemia. These changes appeared to result from increased energy expenditure and activation of the carnitine acyltransferase (CPT) and β -oxidation in WAT. Expressions of the proinflammatory genes *NF- κ B*, *TLR2*, *MCPI*, and *TNF- α* were also lower in the WAT of myricitrin-supplemented mice. Moreover, myricitrin markedly reduced hepatic triglyceride accumulation and plasma aspartate transaminase levels by increasing CPT activity and reducing fatty acid synthase activity in the liver. Myricitrin-supplemented mice also showed improved glucose tolerance, insulin sensitivity, and decreased hyperinsulinemia, along with decreased levels of circulating resistin. In conclusion, long-term consumption of a myricitrin-supplemented diet may effectively protect against HFD-induced obesity and related metabolic disorders.

KEYWORDS: • adiposity • dyslipidemia • hepatic steatosis • inflammation • insulin resistance • myricitrin

INTRODUCTION

OBESITY IS A COMPLEX CONDITION that results from energy imbalance, which is characterized as food intake that exceeds energy requirements over a prolonged period.¹ Adipose tissue is the major site where excess energy is converted to triglycerides and is also considered to be a highly active endocrine organ capable of producing many compounds (known as adipokines) that regulate metabolic homeostasis.² In response to overnutrition, adipose tissue expands by increasing adipocyte size and/or number, and the expanded adipose tissue results in the dysregulation of adipokine secretion, including increased circulating levels of leptin and resistin, which contribute to the adverse health consequences of obesity.³

In addition, excess accumulation of adipose tissue is associated with impaired suppression of free fatty acid release from adipose tissues in response to insulin, which leads to a high concentration of circulating free fatty acids.⁴ Free fatty acids cause insulin resistance and inflammation in the major insulin target tissues (*e.g.*, liver and skeletal muscle), and therefore represent an important link among obesity, inflammation, insulin resistance, and type 2 diabetes.⁴ Notably, abnormally increased delivery of free fatty acids to the liver stimulates the production of hepatic triglycerides, resulting in hepatic steatosis and dyslipidemia.⁴

Myricitrin (3-O- α -L-rhamnopyranoside of myricetin) is a flavonol glycoside that is mainly found in the fruits, branches, bark, and leaves of *Myrica rubra* and other plants. It is well known for its antiallergic,⁵ anti-inflammatory,⁶ antioxidant,⁷ antifibrotic,⁸ and antiatherogenic effects.⁹ Several studies have reported that plants that contain myricitrin may also have beneficial effects on obesity.^{10,11} The myricitrin-containing plant, *Euphorbia lunulata*, inhibits the differentiation of 3T3-L1 preadipocytes and triglyceride accumulation in maturing adipocytes.¹⁰ In addition, aqueous extracts from

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Chrysobalanus icaco leaves that contain myricitrin ameliorate adiposity by increasing locomotor activity, which leads to more energy expenditure and improved insulin sensitivity in high-fat diet (HFD)-induced obese mice.¹¹ Some studies have also demonstrated protective effects of myricitrin, its aglycone, against diet-induced adiposity, insulin resistance, dyslipidemia, and hepatic steatosis in mice or rats.^{12–15} However, there is currently no convincing evidence demonstrating the antiobesity effects of myricitrin.

This study represents the first investigation of whether myricitrin can improve adiposity, inflammation, insulin resistance, dyslipidemia, or hepatic steatosis in HFD-fed C57BL/6J mice, a well-known murine model of the metabolic abnormalities observed in human obesity.¹⁶ We also examined the underlying mechanisms.

MATERIALS AND METHODS

Feeding protocols

C57BL/6J mice ($n=20$, male, 4-week-old; The Jackson Laboratory) were randomly divided into two groups of 10 mice each, and fed *ad libitum* one of the following experimental diets for 16 weeks: an HFD (No. D12451; Research Diets, Inc., New Brunswick, NJ, USA) or an HFD supplemented with myricitrin (0.02%, w/w, ~ 18 mg/kg body weight). The HFD contained 45% kcal as fat, predominantly in the form of lard. Myricitrin was obtained from the bark of *M. rubra*. The barks were extracted with methanol, filtered and concentrated. The methanolic extract was suspended in methanol and filtrated to give a soluble fraction and insoluble residue. The insoluble residue was recrystallized with aqueous methanol to yield myricitrin. Purified myricitrin was identified by spectral analyses (mass spectrometry and nuclear magnetic resonance), which were compared to those of previous reports.¹⁷ Spectroscopic data for purified myricitrin were presented in Supplementary Figure S1.

Food intake was monitored daily during the experiment by weighing the food given, food remaining in the food receptacle, and food dropped to the cage bottom. All measurements were taken at the same time each day. At the end of a feeding period, mice were euthanized after a 12-h fast, and samples (blood, liver, and white adipose tissue [WAT]) were collected as previously described.¹⁸ The animal study protocols were approved by Pukyong National University Ethics Committee (Approval No. 2016-29).

Indirect calorimetry

For energy expenditure calculation, each mouse was allocated to a calorimetry chamber (Oxylet System Metabolic Chambers; Panlab, Cornella, Spain). Recordings of oxygen uptake (V_{O_2}) and carbon dioxide output (V_{CO_2}) over a 24-h period were used to calculate energy expenditure.

Glucose tolerance test and blood parameters analysis

After 15 weeks of feeding on the experimental diets, the glucose tolerance was examined by intraperitoneal glucose

tolerance test (IPGTT) according to a previously described process.¹⁸ Concentrations of plasma insulin, leptin, and resistin were determined using a Multiplex Detection Kit (Cat. No. 171-F7001M; Bio-Rad, Hercules, CA, USA). The levels of fasting blood glucose, plasma glucose, plasma lipids, plasma aminotransferases, and homeostasis model assessment of insulin resistance (HOMA-IR) were determined as previously described.¹⁸

Hepatic lipids contents and lipid-regulating enzymes activities in the liver and epididymal WAT

Hepatic lipids contents were determined using the same commercial kits used for the plasma analyses after extraction.¹⁹ Detailed procedures for isolation of enzyme fraction, determination of protein concentrations, and analyses of fatty acid synthase, carnitine acyltransferase (CPT) and β -oxidation activities were previously described.^{20–24}

Gene expression of inflammatory markers in epididymal WAT

TRIzol Reagent (Invitrogen/Life Technologies, Grand Island, NY, USA) was used for the isolation of total RNA from epididymal WAT. Complementary DNA was synthesized using a QuantiTect Reverse Transcription Kit (Cat. No. 205313; QIAGEN GmbH, Hilden, Germany), and quantitative real-time PCR was carried out on a CFX96TM real-time system (Bio-Rad) using the QuantiTect SYBR Green PCR Kit (Cat. No. 204143; QIAGEN GmbH). Results were analyzed by comparing the Ct value after normalization to control genes (glyceraldehyde 3-phosphate dehydrogenase) using the formula $2^{-\Delta\Delta C_t}$.²⁵

Adipose tissue morphology

Sections of epididymal WAT were stained with hematoxylin and eosin, and then analyzed with an optical microscope (Zeiss Axioscope, Munich, Germany).

Statistical analysis

Data are presented as mean $\pm$ standard error. Student's t -tests were used, and statistical significance was determined at $P < .05$. Statistical analyses were performed using the Statistical Package for Social Science (SPSS) software (version 11.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

Body weight and fat accumulation

Body weight did not differ between the HFD– and HFD+ myricitrin-fed mice during the 16-week feeding period (Fig. 1A). However, myricitrin supplementation significantly decreased the weight of visceral WAT (*i.e.*, the sum of epididymal, perirenal, retroperitoneum, and mesenteric WAT weight) and subcutaneous WAT compared to that of control mice fed an HFD alone, resulting in a significant reduction in total WAT weight (*i.e.*, the sum of the visceral

FIG. 1. Effects of myricitrin supplementation on body weight (A), white adipose tissue weight (B), morphology (C), food intake (D), energy expenditure (E), adipose tissue lipid-regulating enzyme activities (F), and expression of inflammatory genes (G) in C57BL/6J mice fed a high-fat diet. (A, B, D–G) Data are presented as mean $\pm$ SE. Statistical significance between groups was determined using Student t-tests, and is indicated as follows: * P < 0.05, ** P < 0.01. (C) Representative images of H&E-stained epididymal white adipose tissue (original magnification, $\times$ 200). CPT, carnitine palmitoyltransferase; H&E, haematoxylin and eosin; SE, standard error; WAT, white adipose tissue.

and subcutaneous WAT) (Fig. 1B). Moreover, epididymal adipocytes were significantly smaller in the myricitrin group than in the control group (Fig. 1C).

Food intake, energy expenditure, lipid-regulating enzyme activities, and expression levels of proinflammatory markers in the epididymal WAT

Myricitrin supplementation did not alter food intake in HFD-fed mice (Fig. 1D). However, during the night, energy expenditure was significantly increased by myricitrin supplementation (Fig. 1E). Myricitrin also increased daytime energy expenditure by 21% (Fig. 1E). Although no significant differences in fatty acid synthase activity were detected between the two groups, myricitrin significantly increased CPT and β -oxidation activities (Fig. 1F). Moreover, proinflammatory cytokines and mediators (NF- κ B, TLR2, MCP1, and TNF- α) messenger RNA (mRNA) expression was significantly lower in the epididymal WAT of myricitrin group than in the control group (Fig. 1G).

Fasting blood glucose, plasma insulin, HOMA-IR, IPGTT, plasma leptin, and plasma resistin levels

Plasma insulin levels were significantly decreased in the myricitrin group (Fig. 2A), although fasting blood and plasma glucose levels did not differ (data not shown). HOMA-IR was also significantly reduced by myricitrin (Fig. 2B), indicating a reduction in insulin resistance. Moreover, the IPGTT indicated that myricitrin significantly improved glucose tolerance (Fig. 2C). Plasma

leptin and resistin levels were also significantly lower in the myricitrin group than in the control HFD group (Fig. 2D).

Plasma and hepatic lipid levels, hepatic lipid-regulating enzyme activities, and aminotransferases activities

Free fatty acid and triglyceride levels were markedly lowered in the plasma of myricitrin-supplemented mice (Fig. 3A). Moreover, plasma total cholesterol levels were significantly lower in myricitrin-supplemented mice than in control mice, whereas plasma high-density lipoprotein (HDL)-cholesterol levels only showed a nonsignificant trend to be lower (Fig. 3A). As a result of combined reductions in both plasma total cholesterol and HDL-cholesterol levels, ratio of HDL-cholesterol to total cholesterol and the atherogenic index did not differ between the two groups (Fig. 3B, C).

Although there was no significant difference in hepatic cholesterol content, myricitrin significantly reduced hepatic triglyceride levels (Fig. 3D). The activity of hepatic fatty acid synthase, which is a rate-limiting enzyme in fatty acid synthesis,²⁶ was significantly lower in mice that received myricitrin supplementation, while the activity of CPT, a fatty acid oxidation-related enzyme,²⁷ significantly increased in livers of myricitrin-supplemented mice (Fig. 3E). The myricitrin also increased hepatic β -oxidation by 31%, although it was not statistically significant (Fig. 3E). Furthermore, myricitrin significantly decreased plasma aspartate transaminase levels (Fig. 3F). Compared to mice fed a control HFD, mice receiving myricitrin supplementation

FIG. 2. Effects of myricitrin supplementation on plasma insulin levels (A), HOMA-IR (B), glucose tolerance (C), and plasma adipokines levels (D) in C57BL/6J mice fed a high-fat diet. Data are presented as mean ± SE. Statistical significance between groups was determined using Student t-tests, and is indicated as follows: * $P < 0.05$, ** $P < 0.01$. HOMA-IR, homeostasis model assessment of insulin resistance.

had a 28% reduction in plasma alanine transaminase levels, although this difference did not meet our threshold for statistical significance (Fig. 3F).

DISCUSSION

In general, oral bioavailability of flavonoids is low, and gastrointestinal stability is one of the factors that have a significant influence on their oral bioavailability.^{28,29} Myricetin, the aglycone of myricitrin, is also poorly absorbed; absolute bioavailability of myricetin (50 and 100 mg/kg) is about 9% in rats.³⁰ The bioavailability of myricitrin, a rhamnose glycoside of myricetin, appears to take place in the small intestine after hydrolysis of the

glycoside linkage.³¹ However, myricitrin was directly absorbed as the glycosylated form into the intestinal epithelial cells, where they were conjugated and then released into mesenteric blood as glucuronide or sulfate conjugates of myricitrin.³² This was supported by a more recent study.³³ Myricitrin was stable under acidic conditions (pH 1.8) corresponding to the gastric environment and was easily degraded under alkaline intestinal environment (pH 8.5).³⁴ However, degraded myricitrin did not contain myricetin, but still maintained its inhibitory effect on oxidative stress. Another study conducted by Yokomizo and Moriwaki reported that myricitrin was absorbed via the paracellular route, even after degradation in the alkaline intestinal environment.³⁵ In addition, a recent study reported that gastrointestinal stability of myricitrin was higher than myricetin.³⁶ In this study, we assessed whether myricitrin supplementation might influence obesity and obesity-related metabolic disturbances in HFD-induced obese mice.

A recent study reported that myricitrin-containing aqueous extracts of *C. icaco* leaves suppressed adiposity in HFD-fed obese mice, even though it did not affect food intake or body weight.¹¹ The authors of that study suggested that the antiadiposity effect of *C. icaco* leaf aqueous extracts could be related to the presence of polyphenols, such as myricitrin. In support of this idea, we observed that myricitrin decreased both visceral and subcutaneous WATs weight; consequently, total WAT weight was significantly reduced by myricitrin supplementation. Morphological observations also indicated an antiadiposity effect of myricitrin. Similar to the results of a previous study,¹¹ myricitrin did not alter food intake, but it significantly increased energy expenditure, suggesting its possible role in the augmentation of energy expenditure. The ultimate cause of adiposity is an imbalance between energy consumption and expenditure; therefore, increasing energy expenditure has been suggested to be an effective approach to improving obesity and its complications.³⁷

Moreover, myricitrin increased the activity of CPT and β -oxidation in the WAT. The β -oxidation of fatty acids is a multistep process whereby fatty acids are broken down for energy generation. In general, the physiological importance of fatty acid oxidation in WAT has been minimized because the rate of fatty acid oxidation is relatively low in WAT compared with that in other tissues, including liver and skeletal muscle.^{38,39} However, fasting increases fatty acid oxidation in the WAT,^{40,41} and upregulated fatty acid oxidation capacity in the WAT plays important roles in protecting against obesity and its metabolic complications.^{42–44} Impaired fatty acid oxidation resulting from lower *CPT1b* mRNA expression levels in the WAT is suggested to contribute to excessive fat accumulation in obese rats.⁴¹ Pharmacological inhibition of fatty acid oxidation can exacerbate cytokine release and impair insulin activities,⁴³ while upregulation of fatty acid oxidation in 3T3-L1 adipocytes through constitutive overexpression of *CPT1a* results in reduced triglyceride content, insulin resistance, and inflammation.⁴⁴ Therefore, in myricitrin-supplemented obese mice, dietary fat appears to be shunted toward translocation by CPT into mitochondria for β -oxidation and is no longer used for

FIG. 3. Effects of myricitrin supplementation on plasma lipids levels (A), HTR (B), atherogenic index (C), hepatic lipid contents (D), hepatic lipid-regulating enzyme activities (E), and plasma aminotransferases levels (F) in C57BL/6J mice fed a high-fat diet. Data are presented as mean $\pm$ SE. Statistical significance between groups was determined using Student t-tests as follows: * P < 0.05, *** P < 0.001. AST, aspartate transaminase; ALT, alanine transaminase; HTR, ratio of HDL-cholesterol to total cholesterol.

triglyceride synthesis, which may contribute to protection against adiposity. Moreover, we observed that myricitrin improved insulin resistance and downregulated proinflammatory genes expression in the WAT.

WAT is an active endocrine tissue that synthesizes and secretes adipokines and inflammatory cytokines to modulate both inflammation and insulin resistance.³ In obesity, hypertrophic adipocytes exhibit increased expression of proinflammatory cytokines, including $TNF-\alpha$ and MCP1.⁴⁵ Inflammatory activation within the WAT is thought to contribute to obesity-induced insulin resistance along with excess fat accumulation.⁴⁵ $TNF-\alpha$ and MCP1 are known

to be the major WAT-derived inflammatory cytokines responsible for the initiation and progression of insulin resistance.⁴⁶ In HFD-induced obese mice, expression levels of $TNF-\alpha$ and MCP1 were upregulated in WAT,^{45–48} whereas obese mice lacking either $TNF-\alpha$, MCP1, or their respective receptors were protected from the development of insulin resistance.^{47–49} NF- κ B is a transcription factor that controls the expression of these proinflammatory cytokines, and the Toll-like receptor (TLR) family can mediate NF- κ B activation.⁵⁰ It has been reported that the expression levels of TLRs and NF- κ B are upregulated in the WAT of mice with obesity induced by an HFD or leptin deficiency.⁵¹

FIG. 4. Schematic representation of the protective effects of myricitrin on HFD-induced obesity and related metabolic disturbances. The current study demonstrates that flavonoid myricitrin may attenuate adverse metabolic effects of long-term consumption of a high-fat diet, such as adiposity, inflammation, hepatic steatosis, dyslipidemia and insulin resistance, in C57BL/6J mice. These beneficial effects of myricitrin are seemingly attributable to an increase of energy expenditure and regulation of lipid metabolism in adipose tissue and liver. FA, fatty acid; HFD, high-fat diet.

In the present study, myricitrin significantly down-regulated the mRNA expression levels of proinflammatory cytokines, including *TNF- α* and *MCPI*, and of *TLR2* and *NF- κ B* in the epididymal WAT, which may partially contribute to the reduction in insulin resistance. The decreased levels of plasma leptin and resistin observed in myricitrin-supplemented mice could also have contributed to the beneficial effects of myricitrin on adiposity and insulin resistance. Indeed, circulating levels of these adipokines are positively correlated with body fat mass and they have been proposed to play an important role in the pathogenesis of obesity-related insulin resistance.⁵²

Hepatic steatosis, which is characterized by excess fat accumulation in the liver and is a common complication in obesity, has also been related to insulin resistance, although it remains unclear whether it is a cause or consequence of insulin resistance.⁵³ It has been suggested that increased visceral adiposity can result in increased free fatty acid flux directly via the portal vein into the liver, which may in turn increase *de novo* hepatic lipogenesis, trigger very-low-density lipoprotein overproduction and secretion, and impair hepatic insulin clearance. Together, these events would ultimately cause hepatic steatosis, dyslipidemia, and hyperinsulinemia.^{54,55} The present study found that plasma insulin levels were significantly lowered in myricitrin-supplemented mice. Moreover, myricitrin markedly lowered the levels of plasma free fatty acids, triglycerides, and total cholesterol, and also reduced hepatic triglyceride content, suggesting that it exerted protective effects against hepatic steatosis and dyslipidemia.

The reduced levels of plasma aminotransferases in myricitrin-supplemented mice also indicated that myricitrin possibly ameliorated the liver injury induced by HFD. Additionally, in the liver, myricitrin significantly increased CPT activity while inhibiting the activity of fatty acid synthase. Free fatty acids synthesized in the liver and those delivered from the WAT can be either re-esterified to triglycerides within the liver or oxidized via the β -oxidation pathway. In the presence of hyperinsulinemia and insulin resistance, fatty acid esterification is generally favored over oxidation, contributing to the pathogenesis of hepatic steatosis.⁵⁵ Therefore, the amelioration of hepatic steatosis and dyslipidemia observed in myricitrin-supplemented mice may be associated with the inhibition of *de novo* fatty acid synthesis and stimulation of fatty acid oxidation in the liver, along with reduced free fatty acid release from the WAT.

In conclusion, our findings suggest that dietary myricitrin may be an effective antiadiposity agent because of its ability to enhance energy expenditure and fatty acid oxidation in the WAT (Fig. 4). Moreover, it is likely that myricitrin will have the beneficial property of decreasing inflammation in the WAT, along with protecting against dyslipidemia, hepatic steatosis, and insulin resistance (Fig. 4). Considered together, our present findings suggest that myricitrin can ameliorate the deleterious effects of long-term consumption of an HFD, including obesity, plasma lipid abnormalities, hepatic steatosis, inflammation, and insulin resistance.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1

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