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Eriocitrin Improves Adiposity and Related Metabolic Disorders in High-Fat Diet-Induced Obese Mice

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ABSTRACT Eriocitrin (EC) is an abundant flavonoid in lemons, which is known as a strong antioxidant agent. This study investigated the biological and molecular mechanisms underlying the anti-obesity effect of EC in high-fat diet (HFD)-fed obese mice. C57BL/6N mice were fed an HFD (40 kcal% fat) with or without 0.005% (w/w) EC for 16 weeks. Dietary EC improved adiposity by increasing adipocyte fatty acid (FA) oxidation, energy expenditure, and mRNA expression of thermogenesis-related genes in brown adipose tissue (BAT) and skeletal muscle, whereas it also decreased lipogenesis-related gene expression in white adipose tissue. In addition to adiposity, EC prevented hepatic steatosis by diminishing lipogenesis while enhancing FA oxidation in the liver and fecal lipid excretion, which was linked to attenuation of hyperlipidemia. Moreover, EC improved insulin sensitivity by decreasing hepatic gluconeogenesis and proinflammatory responses. These findings indicate that EC may protect against diet-induced adiposity and related metabolic disorders by controlling thermogenesis of BAT and skeletal muscle, FA oxidation, lipogenesis, fecal lipid excretion, glucose utilization, and gluconeogenesis.

KEYWORDS: • ***adiposity*** • ***energy expenditure*** • ***eriocitrin*** • ***fatty acid oxidation*** • ***insulin resistance*** • ***NAFLD***

INTRODUCTION

OBESITY IS ASSOCIATED with metabolic disorders, especially hyperlipidemia, nonalcoholic fatty liver disease (NAFLD), insulin resistance, and type 2 diabetes mellitus. It is at present considered a serious worldwide health risk factor, both for adults and children, and hence represents a serious burden on health care systems.[1] The genesis of obesity is multifactorial, and dysregulated lipid and glucose metabolism in metabolic organs is thought to be a critical factor.[2] An abundance of recent research has reported that energy expenditure (EE) is an important factor in metabolic control of the development of obesity, and that targeting brown adipose tissue (BAT) and skeletal muscle is a promising approach to protect against obesity and its complications in light of their role as thermogenic organs.[3–9]

Most of the studies to date have indicated that certain flavonoids have beneficial effects on obesity and its related diseases such as dyslipidemia, NAFLD, and type 2 diabetes mellitus. Eriocitrin (EC, eriodictyol 7-*O*-β-rutinoside) is an abundant flavonoid in lemons,[2] and its structure is very similar to hesperidin, which has activity against several types of cancer including liver cancer.[10] Recent studies have demonstrated that EC also has antioxidant and antitumor activities, a lipid-lowering effect, and that it improves diet-induced hepatic steatosis.[11–13] Hyperlipidemia and NAFLD are common in obese individuals, and thus we speculated that EC may also have anti-obesity activity. However, the effect of EC on obesity is yet to be elucidated, and the detailed mechanisms by which EC affects the anti-metabolic syndrome are still not understood. Thus, this study was designed to investigate the metabolic actions of EC in high-fat diet (HFD)-induced obese mice. In particular, we focused on its role in controlling EE in BAT and skeletal muscle, and in the amelioration of obesity-associated NAFLD and insulin resistance.

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MATERIALS AND METHODS

Experimental animals and diet

Male 4-week-old C57BL/6N mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA) were individually housed in a pathogen-free barrier facility at room temperature (24°C) and maintained on a 12-h light–dark cycle with water *ad libitum*. After a week of acclimation, the mice were randomly assigned ($n = 10$ mice/group) to either a HFD control group (CON group; 40% fat, 43% carbohydrate, and 17% protein consisting of 85% [w/w] lard and 15% [w/w] corn oil of total fat) or an HFD +0.005% (w/w)

EC group. At the end of the 16th week of EC supplementation, the mice were anesthetized with isoflurane (5 mg/kg body weight; Baxter, Deerfield, IL, USA) after 12 h of fasting, and killed. Blood samples were collected to determine the plasma lipid, adipokine, cytokine, and hormone concentrations. After the blood collection, liver, white adipose tissue (WAT) of epididymal, perirenal, retroperitoneum, subcutaneous and interscapular depots, and interscapular BAT and skeletal muscle were immediately isolated, weighed, snap-frozen in liquid nitrogen, and stored at −70°C until the determination of enzymatic activities and/or RNA analysis. The animal study protocols were approved by the Kyungpook National University Ethics Committee (approval no. KNU 2016-0040, 04/18/2016).

Energy expenditure

EE was assessed using an indirect calorimeter (Oxylet; Panlab, Cornella, Spain) as previously described.[14] The generated data were then averaged for each mouse. EE (kcal/day/kg of body weight$^{0.75}$) was calculated as $VO_2 \times 1.44 \times [3.815 + (1.232 \times VO_2/VCO_2)]$.

Plasma, hepatic, and fecal lipid profile

The plasma free fatty acid (FFA) level was measured using a Wako enzymatic kit (Wako Chemicals, Osaka, Japan). The plasma triglyceride and total cholesterol (TC) concentrations were determined using commercial enzymatic kits (Asan Pharm Co., Seoul, South Korea). After lipid extraction from liver and feces using the classical Folch lipid extraction method,[15] the lipids levels were determined using the same enzymatic kit used for the plasma analyses.

Blood analysis

The levels of plasma insulin, insulin gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1), leptin, resistin, monocyte chemoattractant protein-1 (MCP-1), interferon gamma (IFN-γ), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) were determined using a MILLIPLEX kit (Merck Millipore, Billerica, MA, USA). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated according to the following formula: HOMA-IR = [fasting insulin concentration (mU/L) × fasting glucose concentration (mg/dL) × 0.05551]/22.5. For the glucose tolerance test, at the 15th week after the start of the diet experiments, the mice were fasted for 12 h and then injected intraperitoneally with glucose (0.5 g/kg body weight). Blood glucose levels were measured by piercing the mice tails with a glucose analyzer (OneTouch® Ultra®; Wayne, PA, USA) at 0, 30, 60, and 120 min after glucose injection.

Activities of hepatic lipid- and glucose-regulating enzymes

Hepatic mitochondrial and cytosolic fractions were prepared according to the Hulcher and Oleson method,[16] and protein levels were analyzed using the Bradford method. Cytosolic fatty acid synthase (FAS) and malic enzyme (ME) activities were determined by a spectrophotometric assay according to the method of Carl *et al.* and Ochoa *et al.*, respectively.[17,18] Mitochondrial carnitine palmitoyltransferase (CPT) activity was measured according to the method described by Markwell *et al.*[19] Glucokinase (GK) activity was determined following the method of Davidson and Arion,[20] and phosphoenolpyruvate carboxykinase (PEPCK) activity in the cytosol fraction was measured following the spectrophotometric assay developed by Bentle and Lardy.[21]

RNA isolation and gene expression analysis

Liver, epididymal WAT, interscapular BAT, and gastrocnemius muscle samples were prepared and analyzed as described previously.[22] Total RNA (1 μg) was converted to cDNA using a QuantiTect® reverse transcription kit (QIAGEN GmbH, Hilden, Germany). mRNA expression was quantified by real-time reverse transcription-quantitative polymerase chain reaction (RT-PCR) using the QuantiTect SYBR® Green PCR kit (QIAGEN GmbH) and the CFX96™ real-time system (Bio-Rad, Hercules, CA, USA). The primers used for the real-time RT-PCR are given in Table 1. The cycle threshold values were normalized using *GAPDH*, and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Morphological examination of liver, WAT, and BAT tissues

The livers, epididymal WAT, and interscapular BAT were removed from the mice, fixed in 10% (v/v) paraformaldehyde/phosphate-buffered saline, and embedded in paraffin for hematoxylin and eosin staining. The stained areas were viewed under an optical microscope (Nikon, Tokyo, Japan) at 200× magnification.

Statistical analysis

The values are expressed as mean ± standard error of the mean (SEM). The statistical analyses were carried out using the Statistical Package for the Social Sciences software program (SPSS, Inc., Chicago, IL, USA). Significant differences between the two groups were determined using Student's *t*-test.

RESULTS

EC supplementation reduced body weight and body fat, and regulated the expression of adipocyte genes involved in lipid metabolism

EC supplementation markedly suppressed body weight without altering the food intake (Fig. 1A, B). Moreover, the epididymal adipose size and the weights of the epididymal, retroperitoneal, visceral, subcutaneous, interscapular, and total WAT in the HFD+EC group were significantly decreased compared with those in the HFD-fed CON group (Fig. 1C, D). EC significantly decreased the expression of adipocyte genes involved in lipid uptake (*CD36* and *LPL*) and lipogenesis (*SREBP1*, *ACC*, *FAS,* and *SCD1*), whereas it upregulated the expression of fatty acid (FA) oxidation-

Table 1. Primer Sequences Used for the Reverse Transcription–Quantitative Polymerase Chain Reaction

Gene	*Primer direction*	*Primer sequence*
GAPDH	Forward	5′-CAAGTTCAACGGCACAGTCAAGG-3′
	Reverse	5′-ACATACTCAGCACCAGCATCACC-3′
PPARα	Forward	5′-CCTGAACATCGAGTGTCGAATAT-3′
	Reverse	5′-GGTCTTCTTCTGAATCTTGCAGCT-3′
PPARγ	Forward	5′-GAGTGTGACGACAAGATTTG-3′
	Reverse	5′-GGTGGGCCAGAATGGCATCT-3′
PGC1α	Forward	5′-AAGTGTGGAACTCTCTGGAACTG-3′
	Reverse	5′-GGGTTATCTTGGTTGGCTTTATG-3′
PGC1β	Forward	5′-GGTCCCTGGCTGACATTCAC-3′
	Reverse	5′-GGCACATCGAGGGCAGAG-3′
SREBP1a	Forward	5′-TAGTCCGAAGCCGGGTGGGCGCCGGCGCCAT-3′
	Reverse	5′-GATGTCGTTCAAAACCGCTGTGTGTCCAGTTC-3′
SREBP2	Forward	5′-CACAATATCATTGAAAAGCGCTACCGGTCC-3′
	Reverse	5′-TTTTTCTGATTGGCCAGCTTCAGCACCATG-3′
ACC	Forward	5′-GCCTCTTCCTGACAAACGAG-3′
	Reverse	5′-TGACTGCCGAAACATCTCTG-3′
FAS	Forward	5′-GCTGCGGAAACTTCAGGAAAT-3′
	Reverse	5′-AGAGACGTGTCACTCCTGGACTT-3′
SCD1	Forward	5′-CCCCTGCGGATCTTCCTTAT-3′
	Reverse	5′-AGGGTCGGCGTGTGTTTCT-3′
LPL	Forward	5′-GACTCGCTCTCAGATGCCCTAC-3′
	Reverse	5′-GCCTGGTTGTGTTGCTTGCC-3′
Cd36	Forward	5′-TGGTGGATGGTTTCCTAGCCTTTC-3′
	Reverse	5′-TCGCCAACTCCCAGGTACAATC-3′
ADRB3	Forward	5′-ACCAACGTGTTCGTGACT-3′
	Reverse	5′-ACAGCTAGGTAGCGGTCC-3′
CPT1	Forward	5′-ATCTGGATGGCTATGGTCAAGGTC-3′
	Reverse	5′-GTGCTGTCATGCGTTGGAAGTC-3′
COX8β	Forward	5′-TGTGGGGATCTCAGCCATAGT-3′
	Reverse	5′-AGTGGGCTAAGACCCATCCTG-3′
UCP1	Forward	5′-AGATCTTCTCAGCCGGAGTTT-3′
	Reverse	5′-CTGTACAGTTTCGGCAATCCT-3′
IRS2	Forward	5′-CCCATGTCCCGCCGTGAAG-3′
	Reverse	5′-CTCCAGTGCCAAGGTCTGAAGG-3′
PEPCK	Forward	5′-ATCATCTTTGGTGGCCGTAG-3′
	Reverse	5′-ATCTTGCCCTTGTGTTCTGC-3′
GK	Forward	5′-CAGGACAGTGGAGCGTGAAGAC-3′
	Reverse	5′-TTACAGGGAAGGAGAAGGTGAAGC-3′
UCP3	Forward	5′-GGATTTGTGCCCTCCTTTCTG-3′
	Reverse	5′-AGATTCCCGCAGTACCTGGACT-3′
SLN	Forward	5′-AGCCTACAAGAACAGCAT-3′
	Reverse	5′-GAGGTCCTACCAATACTGAG-3′
SERCA1	Forward	5′-CTGCCGATGATCTTCAAGCTC-3′
	Reverse	5′-CAGGGCACAAGGGCTGGTTAC-3′
SERCA2	Forward	5′-ATCTCCTTGCCTGTGATCCTC-3′
	Reverse	5′-AGTCATGCAGAGGGCTGGTAG-3′

ACC, acetyl-CoA carboxylase; ADRB3, beta-3 adrenergic receptor; CD36, CD antigen 36; COX8*β*, cytochrome c oxidase subunit 8-beta; CPT1, carnitine palmitoyltransferase 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GK, glukokinase; IRS2, insulin receptor substrate 2; LPL, lipoprotein lipase; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, PPAR-gamma coactivator 1-alpha; PPAR, peroxisome proliferator-activated receptor; PPARα, PPAR alpha; PPARγ, PPAR gamma; SCD1, stearoyl-CoA desaturase 1; SERCA, sarco/endoplasmic reticulum Ca2+ ATPase; SLN, sarcolipin; SREBP1α, sterol regulatory element-binding transcription factor 1-alpha; UCP1, uncoupling protein 1.

related genes (*ADRB3*, *CPT1*, *PGC1α*, *PGC1β*, *COX8β,* and *UCP1*) (Fig. 1E).

EC supplementation increased EE and weights of BAT and muscle

The EC group exhibited significantly higher EE during the night and the total 24-h period compared with the mice fed with the HFD (Fig. 2A). In addition, EC supplementation increased the muscle weight, although there was no significant difference, and it greatly increased the expression of the *UCP3*, *SLN*, *SERCA1*, and *SERCA2* genes, which are involved in FA oxidation and thermogenesis, in skeletal muscle (Fig. 2B, C). Moreover, the mice fed with the EC-supplemented diet exhibited a significant increase in the weight of BAT (Fig. 2D). Relative to mice in the CON

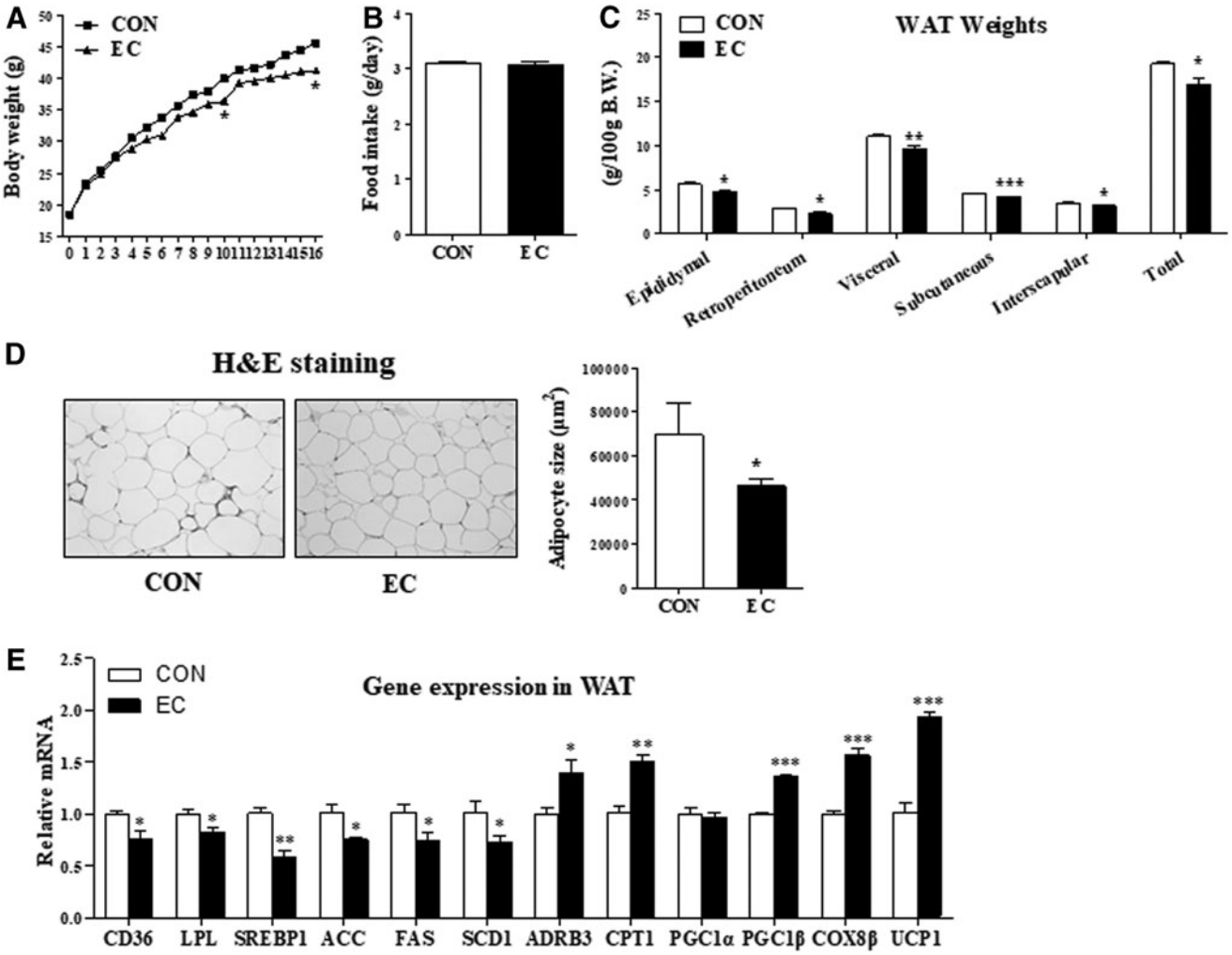

FIG. 1. Effect of EC supplementation for 16 weeks on body weight **(A)**, food intake **(B)**, WAT weights **(C)**, WAT morphology (200× magnification) and adipocyte size **(D)**, and the expression of adipocyte genes **(E)** in HFD-fed mice. The data are presented as mean ± SEM ($n = 10$). The values are significantly different between the groups according to the Student's *t*-test: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, CON versus EC (HFD +0.005% EC). ACC, acetyl-CoA carboxylase; ADRB3, beta-3 adrenergic receptor; CD36, CD antigen 36; CON group, control group; COX8β, cytochrome c oxidase subunit 8β; CPT1, carnitine palmitoyltransferase 1; EC, eriocitrin; FAS, fatty acid synthase; HFD, high-fat diet; LPL, lipoprotein lipase; PGC1, peroxisome proliferator-activated receptor γ coactivator 1; SCD1, stearoyl-CoA desaturase 1; SEM, standard error of the mean; SREBP1, sterol regulatory element-binding transcription factor 1; UCP1, uncoupling protein 1; WAT, white adipose tissue.

group, mice in the EC group exhibited reduced-size lipid droplets with an increase in *UCP1* gene expression in the BAT (Fig. 2E–G).

EC supplementation prevented hepatic steatosis and dyslipidemia, and modulated the expression of hepatic lipid-regulating enzymes and genes

EC-supplemented mice exhibited a markedly reduced size and number of hepatic lipid droplets accumulated around portal vein, and lower hepatic lipid levels of FA and triglyceride, and liver weights, relative to mice in the CON group (Fig. 3A–C). Moreover, EC significantly decreased the expression of lipogenesis-related enzymes (*e.g.*, FAS and ME) and genes (*PPAR*γ and *ACC*), whereas it markedly increased the expression of FA oxidation-related enzymes (CPT) and genes (*PPAR*α and *PGC1*α) in the liver of HFD-fed mice (Fig. 3D, E).

Furthermore, compared with the CON group, the plasma FFA, triglyceride, and TC levels were also decreased by EC supplementation, along with an increase in fecal excretion of FAs and cholesterol (Fig. 3F, G).

EC supplementation improved insulin resistance and glucose tolerance and decreased proinflammatory adipokine/cytokine levels

EC supplementation significantly decreased plasma glucose and insulin levels, and thus markedly reduced HOMA-IR index relative to the mice in the CON group (Fig. 4A–C).

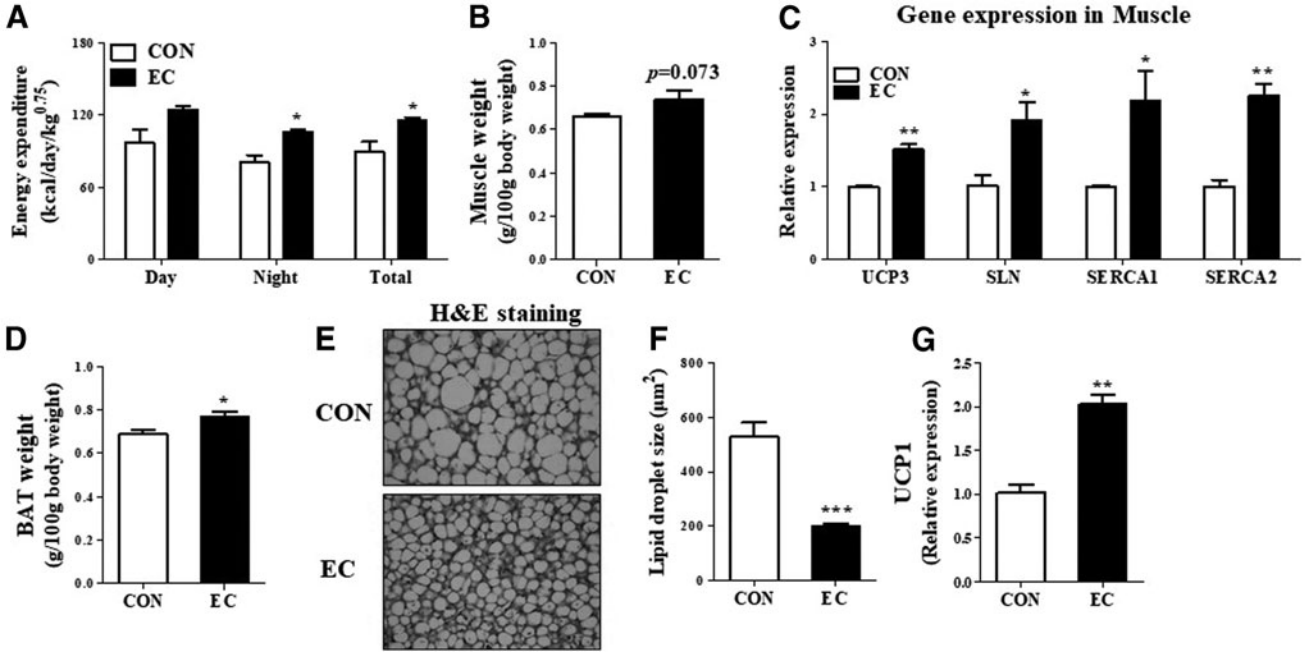

FIG. 2. Effect of EC supplementation for 16 weeks on EE **(A)**, muscle weight **(B)**, the expression of muscle genes **(C)**, BAT weight **(D)**, BAT morphology (200× magnification) **(E)**, size of lipid droplet in BAT **(F)**, and UCP gene expression in BAT **(G)** in HFD-fed mice. The data are presented as mean ± SEM ($n = 10$). The values are significantly different between the groups according to the Student's *t*-test: $^{*}P < .05$, $^{**}P < .01$, CON versus EC (HFD +0.005% EC). BAT, brown adipose tissue; EE, energy expenditure; SERCA, sarco/endoplasmic reticulum Ca2+ ATPase; SLN, sarcolipin.

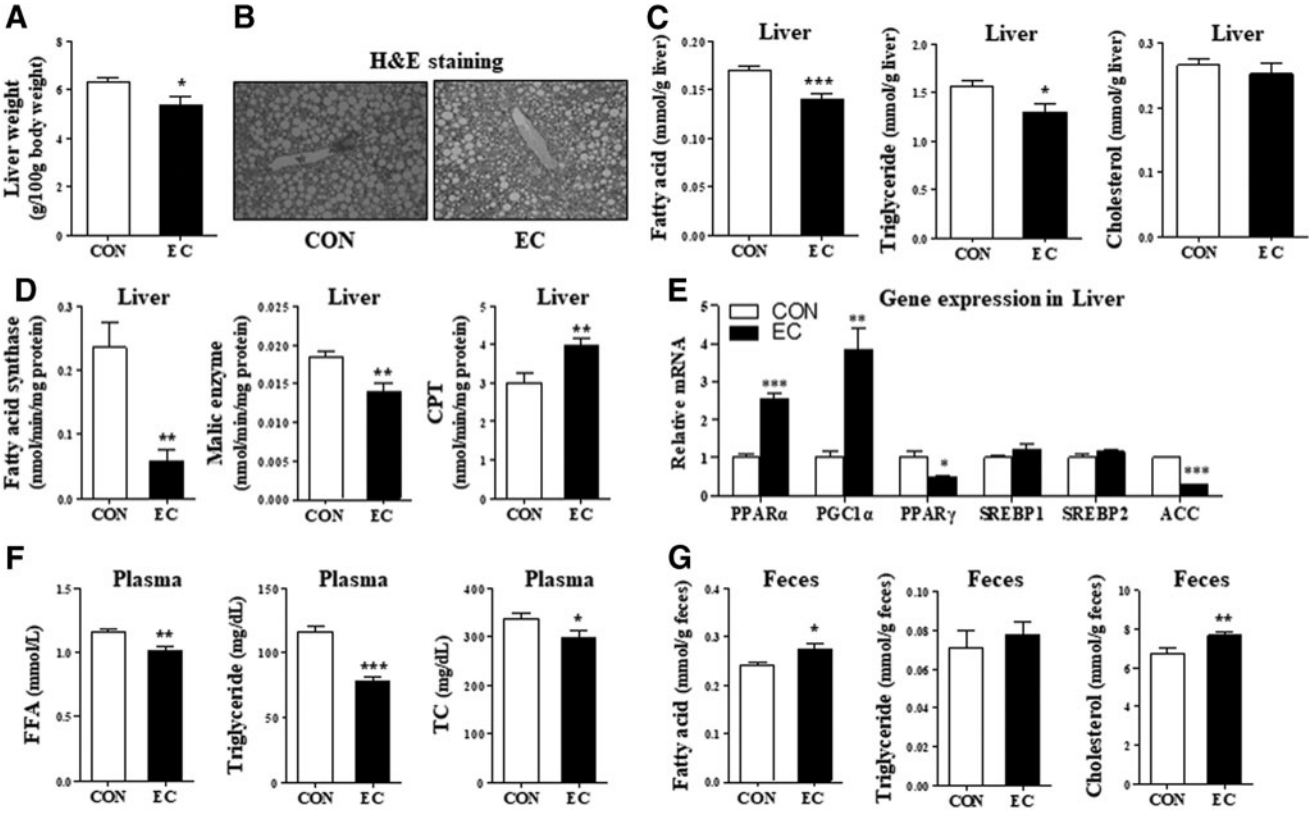

FIG. 3. Effect of EC supplementation for 16 weeks on liver weights **(A)**, hematoxylin and eosin staining of liver (200× magnification) **(B)**, hepatic lipid levels **(C)**, activities of hepatic lipid-regulating enzymes **(D)**, expression of hepatic genes **(E)**, and lipid levels of plasma **(F)** and feces **(G)** in HFD-fed mice. The data are presented as mean ± SEM ($n = 10$). The values are significantly different between the groups according to the Student's *t*-test: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, CON versus EC (HFD +0.005% EC). FFA, free fatty acid; PPAR, peroxisome proliferator-activated receptor; PGC1α, PGC1, peroxisome proliferator-activated receptor γ coactivator 1α.

The intraperitoneal glucose tolerance test also showed that EC supplementation led to improve glucose tolerance in HFD-fed mice (Fig. 4D). In addition, EC supplementation significantly decreased GIP levels, whereas it increased GLP-1 levels in the plasma of HFD-fed mice (Fig. 4E). Moreover, EC supplementation significantly upregulated the enzymatic activity of hepatic GK enzyme and the expression of its gene, which stimulated glycolysis, with an increase in insulin receptor substrate 2 (*IRS2*) gene expression, whereas EC markedly downregulated the enzymatic activity and gene expression of hepatic PEPCK, which increased gluconeogenesis (Fig. 4F, G).

EC-supplemented HFD-fed mice exhibited greatly reduced levels of adipokines (leptin and resistin) and proinflammatory cytokines (MCP-1, IFN-γ, IL-1β, and IL-6) compared with the CON group (Fig. 4H).

DISCUSSION

The mechanism of the development of metabolic syndrome, such as NAFLD and insulin resistance, has recently undergone extensive investigation. Consequently, there is now ample evidence for a strong correlation between metabolic syndrome and obesity, mainly adiposity. In this study, EC dietary supplementation ameliorated adiposity, as evidenced by a decrease in body weight and WAT weight in the absence of alterations of the food intake in HFD-fed mice. The EC-supplemented mice exhibited reduced expression of genes associated with FA uptake (*CD36* and *LPL*) and lipogenesis (*SREBP1*, *ACC*, *FAS*, and *SCD1*), and increased expression of genes involved in FA oxidation (*ADRB3*, *CPT1*, *PGC1β*, *COX8β*, and *UCP1*) in adipose tissue. Previous studies have shown that β3-adrenergic (ADRB3) agonist induced UCP1 expression in WAT.[23] In this study, the enhanced expression of brown fat-specific genes such as *ADRB3*, *PGC1β*, *COX8β*, and *UPC1*, indicators for the browning of WAT, suggested possible conversion WAT to beige adipocytes. WAT browning (brown adipocytes induced in WAT, also known as ''beige'' or ''brite'' cells) may be a promising strategy to combat obesity. Moreover, we observed that EC supplementation significantly increased EE, BAT weight, and UCP1 gene expression in BAT, and a decrease in

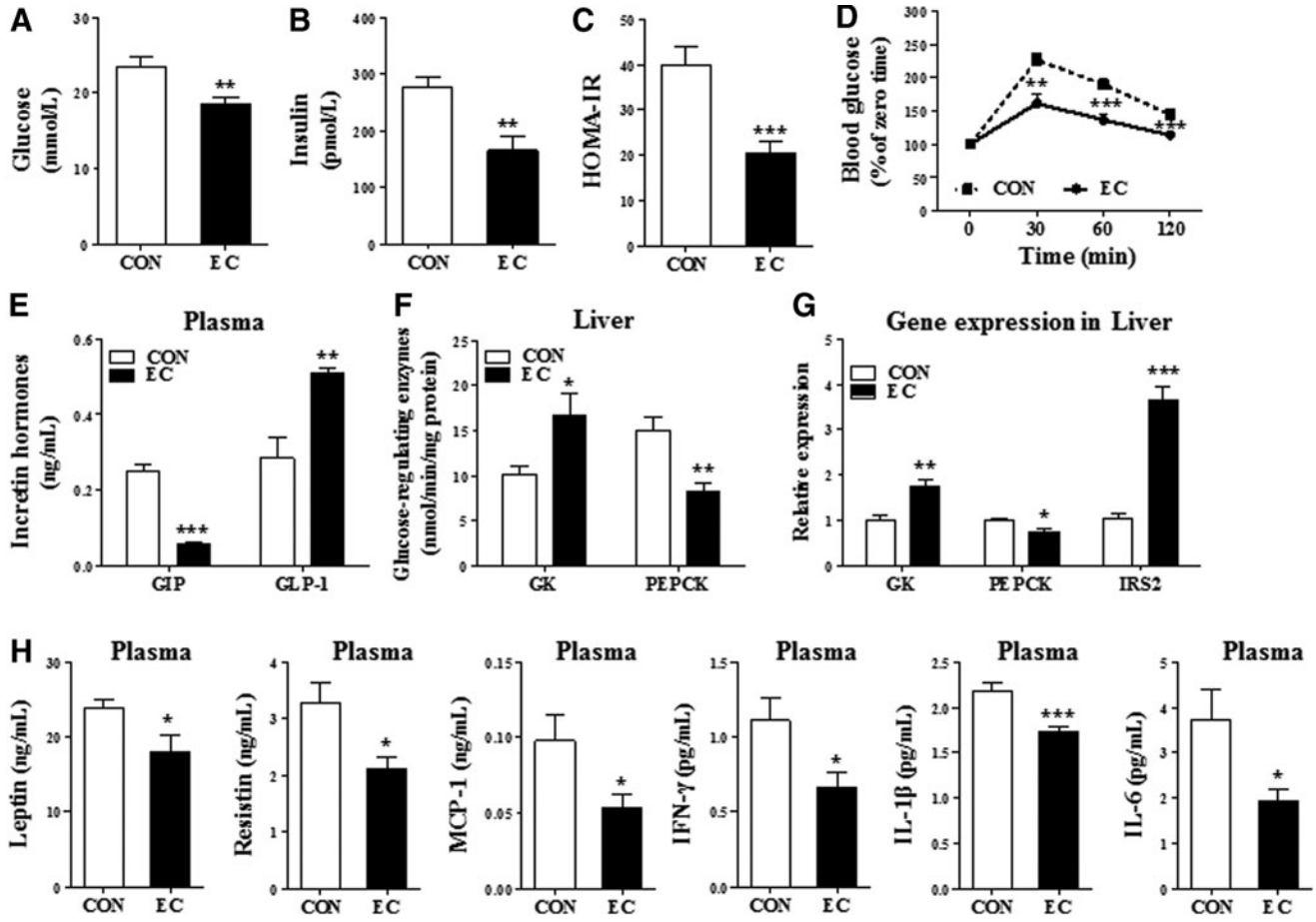

FIG. 4. Effect of EC supplementation for 16 weeks on plasma glucose **(A)**, insulin **(B)**, HOMA-IR **(C)**, glucose tolerance **(D)**, incretin hormones **(E)**, hepatic glucose-regulating enzyme activities **(F)**, hepatic gene expressions **(G)**, and plasma adipokine/cytokine levels **(H)** in HFD-fed mice. The data are presented as mean ± SEM ($n = 10$). The values are significantly different between the groups according to the Student's *t*-test: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, CON versus EC (HFD +0.005% EC). GIP, gastric inhibitory polypeptide; GK, glucokinase; GLP-1, glucagon-like peptide-1; HOMA-IR, homeostasis model assessment of insulin resistance; IFN-γ, interferon γ; IL, interleukin; IRS2, insulin receptor substrate 2; MCP-1, monocyte chemoattractant protein-1; PEPCK, phosphoenolpyruvate carboxykinase.

lipid droplets in BAT in mice fed an HFD. There is evidence that reduced EE and FA oxidation to utilize fat for metabolic fuel are important risk factors for the development of obesity and its complications.[24] Activation of nonshivering thermogenesis contributes to increased EE, thereby counteracting obesity, insulin resistance, and diabetes. One such mechanism is the activation of thermogenesis in BAT, and BAT can be recruited to increase EE by mediation of thermogenesis in response to HFD.[3–5] In addition, several studies have highlighted that skeletal muscle is also an important player in adaptive thermogenesis.[6–7] Enhancing SLN activity, which is a positive regulator of the SERCA pump, increased adenosine triphosphate (ATP) hydrolysis, and heat production in skeletal muscle could thus be a potential therapeutic target to increase EE and to control weight gain.[8,9] In this study, EC supplementation enhanced expression of genes involved in heat production such as *UCP3*, *SLN*, *SERCA1*, and *SERCA2,* in skeletal muscle, with an increase in muscle weight, which may be related to the increased EE and concomitant decrease in adiposity in the HFD-fed obese mice. Consequently, the coordinated action of decreased FA uptake and lipogenesis in WAT, increased thermogenesis in both BAT and skeletal muscle, and enhanced EE seem to have contributed to the beneficial effect of EC supplementation on adiposity in the HFD-induced obese mice.

Next, we investigated the effect of EC supplementation on NAFLD. NAFLD, which encompasses a range of conditions caused by lipid deposition within liver cells, is related to metabolic disorders, especially obesity, hyperlipidemia, insulin resistance, and type 2 diabetes.[25] Regulation of lipid metabolism in the liver is crucial for preventing the development of NAFLD, and there is increasing evidence that dysregulated lipid metabolism such as decreased FA oxidation and enhanced lipogenesis in liver as a result of obesity increases not only NAFLD but also insulin resistance.[26,27] Moreover, NAFLD can result in the development of nonalcoholic steatohepatitis, which is a severe form of NAFLD caused by chronic inflammation and fibrogenesis, which can progress to cirrhosis and hepatocellular carcinoma.[26] In this study, EC supplementation decreased the accumulation of lipid droplets and the lipid content (FA and triglyceride) in liver, and it also reduced liver weights, suggesting an improvement of NAFLD. These effects may be partially involved in the regulation of the expression of hepatic lipid-regulating enzymes or hepatic genes. The activity

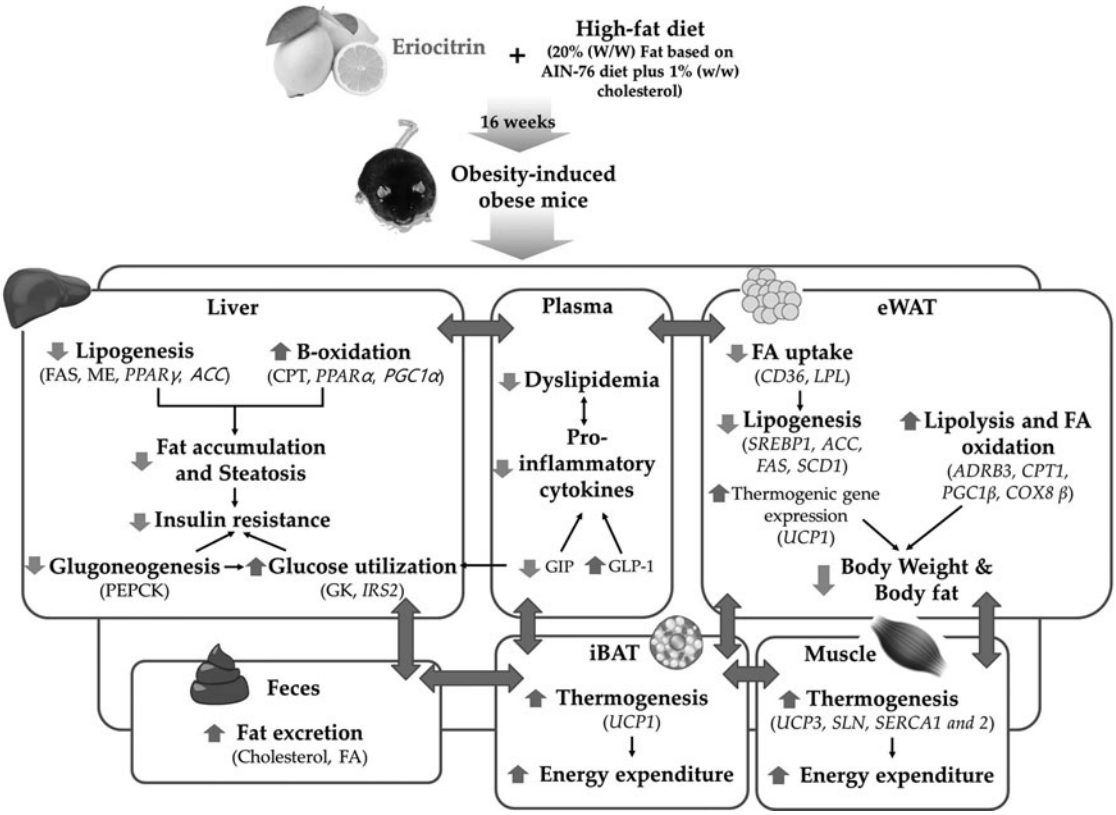

FIG. 5. Proposed role of flavonoid EC in attenuating adiposity, hepatic steatosis, insulin resistance, and inflammation in high-fat fed mice. In HFD-induced obese mice, dietary EC supplementation improved adiposity by increasing adipocyte FA oxidation, EE, and mRNA expression of thermogenesis-related genes in BAT and skeletal muscle, whereas it also decreased lipogenesis-related gene expression in WAT. In addition to adiposity, EC alleviated hepatic steatosis by decreasing hepatic lipogenesis while increasing hepatic FA oxidation and fecal lipid excretion, which was linked to attenuation of hyperlipidemia. Moreover, EC improved insulin resistance, in part, by promoting glucose utilization, by decreasing hepatic gluconeogenesis and proinflammatory responses, and by modulating the production and release of two incretin hormones, namely, GIP and GLP-1. These findings indicates that EC may protect against diet-induced adiposity and related metabolic disorders by controlling thermogenesis of BAT and skeletal muscle, FA oxidation, lipogenesis, fecal lipid excretion. FA, fatty acid.

of hepatic FAs and ME, and *PPARγ* and *ACC* mRNA expression, which regulate lipogenesis, were significantly decreased in the EC-treated mice. EC supplementation also markedly increased the activity of CPT and the expression of *PPARα* and *PGC1α* mRNA in liver of the HFD-fed mice, thereby increasing FA oxidation. These results are in accordance with a previous study that demonstrated that supplementation with EC for 4 weeks protected against hepatic steatosis by increasing hepatic *PPARα* and other *β*-oxidation enzyme genes, *acox1* and *acadm*, in zebrafish with diet-induced obesity.[13] Moreover, supplementation of EC significantly decreased plasma lipid levels (FFA, triglyceride, and TC) while also markedly increasing fecal excretion of lipids (FA and cholesterol) in HFD-fed mice. These findings suggest that EC ameliorated NAFLD in HFD-fed mice by limiting hepatic lipid availability by activation of FA oxidation and fecal excretion of lipids, and by decreasing *de novo* FA synthesis, which is partially linked with the attenuation of hyperlipidemia by EC supplementation.

Insulin resistance is the pathophysiological hallmark of NAFLD. In addition to improving adiposity and NAFLD, EC supplementation prevented insulin resistance. The EC group exhibited a significant decrease in fasting plasma glucose, insulin, and HOMA-IR levels, and improved glucose tolerance compared with the HFD group. Furthermore, relative to the HFD mice, EC supplementation not only significantly decreased the level of circulating GIP, which facilitates fat deposition in adipose tissue and a high level of GIP has been reported to cause insulin resistance,[28] but it also markedly increased the plasma level of GLP-1, which can exert anti-inflammatory effects.[29] Dysregulated hepatic glucose metabolism caused by increased gluconeogenesis and decreased glycolysis has also been reported to contribute to the onset of the insulin resistance.[30,31] Therefore, enhanced hepatic glucose utilization by the increased expression of hepatic GK and *IRS2* mRNA, and the suppression of gluconeogenesis by the decreased expression and lower enzymatic activity of hepatic PEPCK seems to be associated with the improved insulin sensitivity observed in EC-supplemented obese mice, which is similar to what was found in a previous study.[22] In addition, we determined the levels of plasma adipokines and cytokines to investigate the anti-inflammatory effect of EC supplementation. Our results indicate that EC supplementation decreases the plasma level of proinflammatory cytokines (MCP-1, IFN-*γ*, IL-1*β*, and IL-6), and adipokines (leptin and resistin), thus suggesting that EC may have a protective role in chronic low-grade inflammation, which contributes to the development of insulin resistance and NAFLD.[32]

In conclusion, our observations using diet-induced obese mice demonstrate a novel mechanism of EC action in improving obesity and related metabolic disorders such as hyperlipidemia, NAFLD, and insulin resistance. Dietary EC improved adiposity by increasing adipocyte FA oxidation, EE, and mRNA expression of thermogenesis-related genes in BAT and skeletal muscle, and by decreasing the expression of lipogenesis-related genes in WAT (Fig. 5). EC supplementation also decreased hepatic lipogenesis and prevented hyperlipidemia, whereas it increased hepatic FA oxidation and fecal lipid excretion, thus suggesting amelioration of HFD-induced hepatic steatosis. Moreover, EC supplementation improved insulin resistance, as evidenced by reduced HOMA-IR and improved glucose tolerance, and it decreased hepatic gluconeogenesis and proinflammatory responses. Taken together, the present findings indicate that EC may be useful for ameliorating the deleterious effects of HFD-induced obesity and related metabolic complications such as hyperlipidemia, NAFLD, and insulin resistance.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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