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Ellagic acid improves hepatic steatosis and serum lipid composition through reduction of serum resistin levels and transcriptional activation of hepatic *ppara* in obese, diabetic KK-A^y mice

Yukihiro Yoshimura, Saori Nishii, Nobuhiro Zaima, Tatsuya Moriyama *, Yukio Kawamura

Department of Applied Biological Chemistry, Graduate School of Agricultural Science, Kinki University, 204-3327 Nakamachi, Nara City, Nara 631-8505, Japan

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ABSTRACT

Ellagic acid (EA) is a polyphenol found in a wide variety of plant foods that not only exhibits free radical-scavenging activity, but also confers protective effects against liver injury. Previously, we reported that pomegranate fruit extract (PFE) had an inhibitory effect on resistin secretion from differentiated murine 3T3-L1 adipocytes and identified EA contained in PFE as a potent suppressor of resistin secretion. Resistin, an adipocytokine, is considered the link between obesity and type 2 diabetes. In this study, we explored whether EA supplementation reduces serum resistin and improves hepatic steatosis and serum lipid profile by using KK-A^y mice fed high-fat diet as a model for obese type 2 diabetes. We found that EA supplementation improved serum lipid profile and hepatic steatosis, and reduced serum resistin levels without altering mRNA expression levels in adipose tissue. Moreover, EA supplementation upregulated mRNA expression of *apoa1*, *ldlr*, *cpt1a*, and *ppara* genes in the liver. In conclusion, our findings indicate that EA is a potent suppressor of resistin secretion *in vivo* and a transcriptional activator of *ppara* in the liver, suggesting a possibility for improving obesity-induced dyslipidemia and hepatic steatosis in KK-A^y mice.

1. Introduction

Metabolic syndrome is a complex disorder characterized by visceral obesity, hypertension, dyslipidemia, and an impairment of glucose metabolism, which is a major risk factor for the development of cardiovascular disease and/or type 2 diabetes [1,2]. One of the major causes of metabolic syndrome is dysregulation of adipocytokines secreted from adipose tissue [3,4], as the cytokines play a central role in energy and vascular homeostasis as well as in immunity. Of these, adiponectin, has beneficial roles such as anti-steatosis [5], anti-inflammation [6], and improving insulin sensitivity [7]. Low levels of serum adiponectin are a diagnostic marker for metabolic syndrome, atherosclerosis, and non-alcoholic fatty liver disease [8–10]. In contrast to adiponectin, resistin has unfavorable effects on metabolic syndrome. There exists a difference between rodents and humans in terms of expression levels of resistin in tissues. In contrast to rodents which express resistin mainly in adipose tissue, resistin expression in humans is found mainly in bone marrow and in macrophages, and only in low levels in adipose tissue [11,12]. Apart from their different resistin expression patterns, concentrations of serum resistin may be related to metabolic syndrome in both human and rodents. Elevated levels of plasma resistin were observed in humans with obesity and type 2 diabetes [13]. In mice, resistin overexpression leads to dyslipidemia characterized by high concentrations of serum total cholesterol and triglyceride (TG), reduced high-density lipoprotein cholesterol (HDL-C) concentration, and increased low-density lipoprotein cholesterol concentration, which is a common feature in metabolic syndrome [14]. Conversely, ablation of the *resistin* gene improves insulin sensitivity in mice [15,16], and also hyperlipidemia and hepatic steatosis in *leptin*-deficient mice [17,18]. These studies aimed at understanding the biological roles of resistin in human and rodents allow us to search for compounds to suppress elevated serum resistin levels in order to prevent or improve the symptoms of metabolic syndrome.

Recently, we found that administration of pomegranate fruit extract (PFE) reduced serum resistin in ovariectomized mice that show high concentrations of serum resistin. We also identified ellagic acid (EA), a component of PFE as the active compound which suppresses resistin secretion from differentiated murine 3T3-L1 adipocytes without altering resistin mRNA expression [19]. EA is a polyphenol found in a wide variety of plant foods such as grapes and pomegranates [20,21]. EA has free radical scavenger activity and showed anti-proliferative effects on various types of cancer cell lines *in vitro* and *in vivo* [22,23]. Furthermore, EA showed protective effects against liver injury induced by cisplatin [24] and carbon-tetrachloride [25] in rodents, and also attenuated high-carbohydrate, high-fat diet-induced metabolic syndrome character-

* Corresponding author. Fax: +81 742 43 8070.
E-mail address: tmoriyama@nara.kindai.ac.jp (T. Moriyama).

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ized by liver inflammation in rats [26]. These studies and our recent study [19] suggest that EA has potent beneficial effects on metabolic syndrome by improving dysregulation of resistin and/or liver functions.

In this study, we fed a high-fat diet, which included EA, to KK-A^y mice as an animal model with metabolic syndrome/type 2 diabetes to investigate the effects of EA on serum resistin levels and liver functions *in vivo*.

2. Materials and methods

2.1. Animal experiments

KK-A^y mouse (CLEA, Tokyo, Japan) is combined models made by introduction of the obesity gene A^y into KK mouse, which develops obesity and hyperglycemia at an earlier stage. Four-week-old male KK-A^y mice were housed individually and maintained on a standard 12-h light–dark cycle. After acclimatization for a week, they were divided into 2 groups: the control group was fed high-fat diet (HF, Supplementary Table 1) only, and the EA group received the HF containing 0.1% EA (Wako Pure Chemical Industries, Osaka, Japan) for 68 days. Food and water were given *ad libitum* to each group. All animal experiments were approved by the Institutional Animal Care and Use Committee and carried out according to the Kinki University Animal Experimentation Regulations.

At day 45, mice were starved for 17 h and then blood samples were collected from tail vein to measure their fasting blood glucose level. At the end of the treatment period, the mice were sacrificed under anesthesia with pentobarbital and their abdominal adipose tissue (epididymal, perirenal, and mesentery white adipose tissue (WAT)) and liver weight were measured. Blood samples were collected, and serum was obtained by centrifugation at 3000×*g* for 10 min. The serum levels of glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), TG, free fatty acid (FFA), total cholesterol, and HDL-C were determined by enzymatic assay (Wako Pure Chemical Industries). Serum concentrations of non-HDL-C cholesterol were calculated by subtracting HDL-C concentrations from total cholesterol. The serum levels of adiponectin and resistin were measured using ELISA kits (R&D Systems, Minneapolis, MN) and those of leptin were measured by using a Leptin ELISA Kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

2.2. Measurement of triglyceride contents in liver

TG was extracted from liver with chloroform/methanol by the Bligh and Dyer method [27] and TG concentration was measured using an enzymatic assay kit described above.

2.3. Histological analysis of the liver

Individual livers were harvested and frozen immediately in liquid nitrogen. Frozen tissue sections of 10-μm thickness were prepared using a cryostat (CM 1850; Leica Microsystems, Wetzler, Germany). Cryosections were fixed 4% paraformaldehyde for 10 min, and then stained with Mayer's hematoxylin and eosin (HE) or with Oil Red O and Mayer's hematoxylin.

2.4. Quantification of mRNA expression levels

Total RNA was prepared from the liver and epididymal WAT by using Sepasol-RNA super I (Nacalai Tesque, Kyoto, Japan) and reverse-transcribed by using PrimeScript® RT reagent Kit (Takara Bio, Shiga, Japan). To determine mRNA expression levels, real-time quantitative PCR analyses were performed by using the Thermal Cycler Dice™ Real Time System (TP8000; Takara Bio) with SYBR® Premix Ex Taq™ (Takara Bio) as described previously [19]. The primer sequences used for the amplification were described in Supplementary Table 2. Gene expressions in the epididymal WAT and liver were normalized to *gapdh* and *actb* expressions, respectively.

2.5. Statistical analysis

Data represent mean ± SE and each of animal experiments was analyzed by Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of EA supplementation on physiological and metabolic parameters in obese KK-A^y mice

To investigate the effects of EA on type 2 diabetes mellitus, we fed EA to KK-A^y mice along with the high-fat diet. Table 1 presents the effects of EA on physiological and metabolic parameters in KK-A^y mice. At the end of administration, no significant differences were found in body weight, visceral fat weight from either of the sources (epididymal, perirenal, and mesentery WAT), total visceral fat weight, and liver weight between control and EA groups. Fasting glucose value of EA group was significantly lower than that of control group. On the other hand, serum glucose at the end-point was not changed by EA supplementation, and serum insulin concentration tended to be lower in EA group compared with control group. The results of fasting glucose and insulin concentrations suggest that EA supplementation improved diabetic condition in KK-A^y mice, while long-term supplementation of EA to obese KK-A^y mice affected their serum lipid profile. Serum concentrations of FFA and TG in the EA group were significantly lower than those in control group were. Serum total cholesterol in EA group tended to be lower than that of the control group. On the other hand, serum HDL-C content was significantly higher in EA group than that in the control group. In addition, non-HDL-C concentration in serum was significantly lower in EA group than that in control group. These results indicate that EA supplementation improved serum lipid composition in KK-A^y mice. In addition to serum lipid profile, EA ameliorated the increased serum AST and ALT activities, sug-

Table 1
Effects of EA on physiological variables and serum biochemistry.

	Control	Ellagic acid	*p* Value
Initial body weight (g)	25.8 ± 0.5	25.4 ± 0.5	0.57
Final body weight (g)	51.9 ± 1.1	51.1 ± 1.5	0.66
Body weight gain (g)	26.0 ± 0.8	25.6 ± 1.1	0.77
Epididymal WAT(g)	1.8 ± 0.1	2.0 ± 0.1	0.17
Perirenal WAT (g)	0.7 ± 0.1	0.8 ± 0.1	0.16
Mesentery WAT (g)	1.4 ± 0.1	1.3 ± 0.1	0.37
Total WAT (g)	3.9 ± 0.1	4.2 ± 0.2	0.35
Liver weight (g)	5.9 ± 0.3	5.4 ± 0.3	0.28
Fasting glucose (mmol/L)	3.5 ± 0.3	2.9 ± 0.1	<0.05
Serum glucose concentration (mmol/L)	17.3 ± 1.3	16.9 ± 1.3	0.24
Serum insulin concentration (μg/L)	17.7 ± 2.8	13.8 ± 2.3	0.30
Serum AST (U/L)	32.3 ± 1.9	24.1 ± 2.7	<0.03
Serum ALT (U/L)	11.6 ± 2.5	6.3 ± 1.5	0.09
Serum total cholesterol (mg/dL)	301.6 ± 16.4	272.0 ± 12.7	0.17
Serum HDL cholesterol (mg/dL)	104.4 ± 5.7	129.7 ± 6.0	<0.008
Serum non-HDL cholesterol (mg/dL)	197.2 ± 11.4	142.3 ± 11.1	<0.004
Serum free fatty acid (mmol/L)	1.8 ± 0.04	1.4 ± 0.07	<0.0003
Serum triglyceride (mg/dL)	180.5 ± 13.3	138.1 ± 11.6	<0.03

Values are expressed as mean ± SE ($n = 9$). WAT; white adipose tissue; AST, aspartate aminotransferase; ALT; alanine aminotransferase; HDL; high-density lipoprotein.

gesting that EA supplementation also improved the conditions associated with liver injury in KK-A^y mice.

3.2. EA supplementation improved hepatic steatosis and triglyceride contents in obese KK-A^y mice

The liver is one of the major sites of lipogenesis, which encompasses fatty acid synthesis and subsequent TG synthesis, and synthesizes the various lipoproteins involved in transporting cholesterol and lipids throughout the body. EA supplementation in obese KK-A^y mice improved their serum lipid profile consisting of FFA, TG, and cholesterol, and also improved the increase of ASL/ ALT activities which indicates liver tissue damage (Table 1), suggesting that EA affected the liver. To investigate the effects of EA supplementation on the liver in KK-A^y mice, we next prepared the liver sections. Histological sections of the liver from control group showed severe macrovesicular steatosis (Fig. 1A), whereas that from EA group showed mild microvesicular steatosis (Fig. 1B). Oil red O staining of liver sections clearly showed that hepatic steatosis in KK-A^y was improved by EA supplementation (Fig. 1C, D). Furthermore, liver TG contents in EA group were significantly lower than that in control group (Fig. 1E). These results suggest that EA supplementation improved TG accumulation in the liver, which subsequently reduced serum TG contents.

3.3. EA supplementation reduced serum level of resistin without altering resistin mRNA expression level in obese KK-A^y mice

Adipocytokines secreted from adipose tissue regulate metabolism and insulin resistance, thus contributing to chronic inflammation associated with metabolic syndrome. Resistin was originally reported as an adipocytokine, which provided a link between obesity and diabetes [28]. In rodents, resistin is secreted mainly from the adipose tissue, which is different from that in humans where secretion of resistin is mainly seen in macrophages [11,12]. Gene ablation of resistin induced in *leptin*-deficient *ob/ob* mice improved hepatic steatosis and serum lipid profile [18]. These findings indicate that resistin is involved not only in insulin resistance, but also in the exacerbation of lipid metabolism in the liver and blood. Recently, we found that EA inhibits resistin secretion from differentiated mouse 3T3-L1 adipocytes without altering resistin mRNA expression, and reduces serum resistin in ovariectomized mice which usually show high concentration of serum resistin [19]. To investigate the mechanisms underlying the improvement of hepatic steatosis and serum lipid profile in KK-A^y mice by EA supplementation, we measured levels of some of the adipocytokines in serum. Similar to ovariectomized mice [19], serum resistin levels in the EA group were significantly lower than those in control group were (Fig. 2A). To confirm our recent finding that EA inhibits resistin secretion without altering resistin gene transcription *in vivo*, we measured mRNA expression of resistin in WAT. As expected, resistin mRNA expression in WAT was not affected by EA supplementation (Fig. 2B); at the same time, EA supplementation did not show any effects on serum adiponectin and leptin levels in KK-A^y mice (Fig. 2C, D). These data suggested that EA supplementation affected resistin secretion specifically from the adipose tissue *in vivo*.

3.4. EA supplementation increased mRNA expression of PPARα and expression of genes involved in lipid metabolism and serum cholesterol levels in the liver

To further investigate the mechanisms underlying improvement of hepatic steatosis in KK-A^y mice by EA supplementation, we measured expression levels of mRNA involved in fatty acid metabolism and cholesterol transport in the liver. In addition to TG content in the liver, serum levels of HDL-C and non-HDL-C improved in the EA group (Table 1). To understand the mechanisms underlying this, we measured the mRNA expression levels of genes in the liver involved in cholesterol synthesis and transport. The transcriptional levels of *srebf2* (sterol regulatory element-binding transcription factor 2), *apoa1* (apolipoprotein A–I), and *ldlr* (low-density lipoprotein receptor) were significantly higher than the control group (Fig. 3A), suggesting that the increased expressions of ApoA1 and LDL receptor in the liver by EA supplementation leads to the improved serum cholesterol profile. Although it was unclear whether cholesterol content in the liver of the EA group was lower compared to that of control group, the up-regulation of *srebf2* might cause the maintenance of cholesterol homeostasis in the liver. In fatty acid synthesis-related genes, the mRNA expression of *fasn* (fatty acid synthase) was significantly upregulated up to 2.3-fold in EA group compared with that in control group (Fig. 3B). Although the expression of *fasn* was upregulated, the mRNA expression of *acaca* (acetyl-CoA carboxylase alpha) which is the rate limiting enzyme in fatty acid synthesis and *srebf1c* (sterol regulatory element-binding transcription factor 1c), which regulates the transcriptional levels of many genes involved in fatty acid synthesis, were not changed by EA supplementation

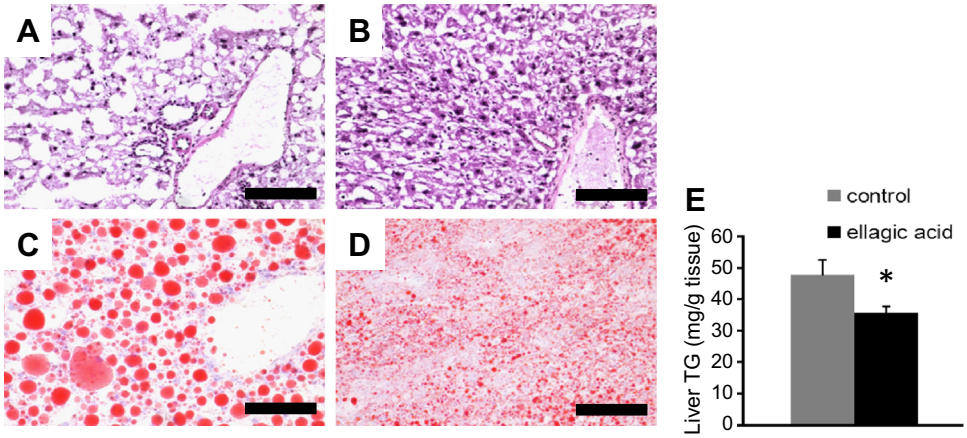

Fig. 1. EA ameliorated the fatty liver and liver triglyceride contents in the obese KK-A^y mice. (A, B) Hematoxylin and eosin staining of the control mouse liver (A) and EA-fed mouse liver (B). (C, D) Oil red O staining of the control mouse liver (C) and EA-fed mouse liver (D). Scale bar shown in each panel indicates 50 μm. (E) Liver triglyceride contents in obese KK-A^y mice. Values are expressed as mean ± SE (control, $n = 8$; EA, $n = 9$). $^*p < 0.05$ compared with control mice.

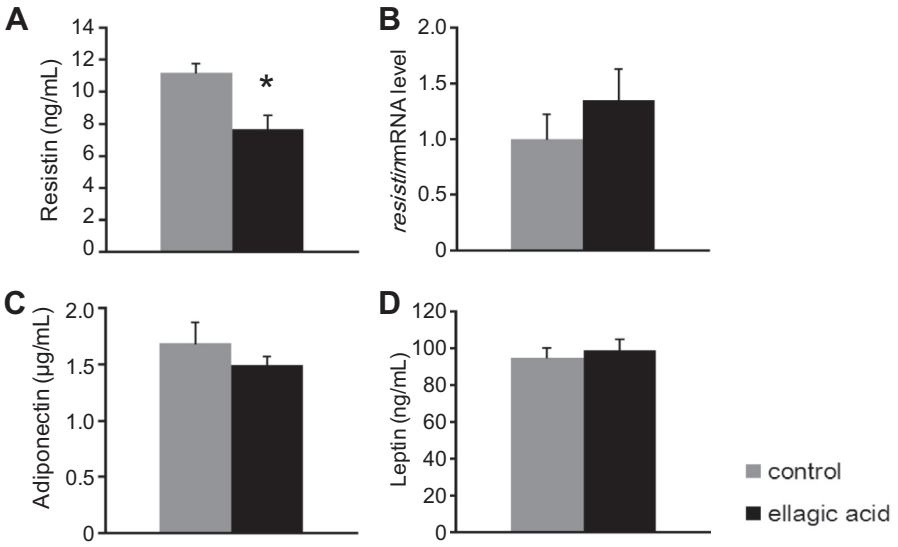

Fig. 2. EA reduced serum resistin levels in obese KK-A^y mice without altering resistin transcriptional levels in white adipose tissue. (A–C) Effects of EA on serum adiponectin (A), leptin (B) and resistin (C) levels in obese KK-A^y mice. (D) Resistin mRNA expression levels of in white adipose tissue of KK-A^y mice measured by quantitative RT-PCR. The relevant amount of resistin transcript was normalized to the amount of *gapdh* transcript. Values are expressed as mean ± SE ($n = 9$). $^{*}p < 0.005$ compared with control mice.

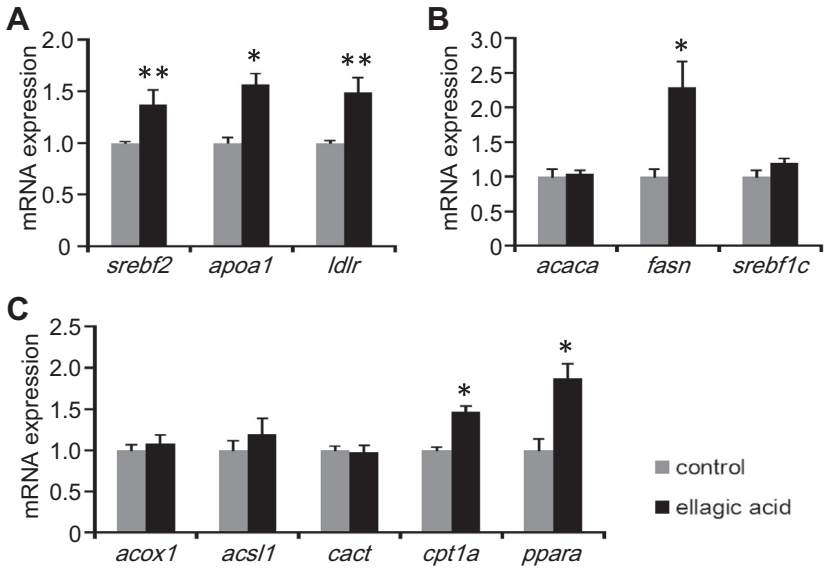

Fig. 3. Effects of EA supplementation on mRNA expression levels of genes involved in lipid metabolism in the liver of obese KK-A^y mice. The mRNA expression levels of genes involved in cholesterol metabolism (A), fatty acid synthesis (A) and metabolism (C) measured by quantitative RT-PCR. The respective amounts of each transcript were normalized to the amount of *actb* transcript. Values are expressed as mean ± SE ($n = 5$ or 6). $^{*}p < 0.01$ and $^{**}p < 0.05$ compared with control mice.

(Fig. 3B). In the fatty acid oxidation-related genes, the mRNA expression levels of *acox1* (acyl-CoA oxidase 1), *acsl1* (long-chain-fatty-acid–CoA ligase 1) and *cact* (carnitine-acylcarnitine translocase) were not changed in both groups (Fig. 3C). On the other hand, the transcriptional level of *cpt1a* (carnitine palmitoyltransferase 1A) which is rate limiting enzyme of fatty acid β-oxidation in was significantly upregulated by 1.5-fold in the EA group compared with that of control group (Fig. 3C). Interestingly, the mRNA expression of *ppara* (peroxisome proliferator-activated receptor alpha) regulating the transcriptional levels of many important genes involved in fatty acid β-oxidation was significantly upregulated by 1.9-fold in EA group compared with that in control group (Fig. 3C). These results in Fig. 3B and C suggest that fatty acid synthesis was not increased, whereas fatty acid degradation occurred actively in the liver of EA group.

4. Discussion

In this study, we investigated effects of EA on serum resistin levels and liver functions in KK-A^y mice. EA supplementation reduced serum resistin levels but not that of other adipocytokines such as adiponectin and leptin, and improved hepatic steatosis and serum lipid profile in high-fat fed obese, diabetic KK-A^y mice. Recently, we identified EA as a specific inhibitor of resistin secretion from differentiated 3T3-L1 adipocytes [19]. Although resistin secretion was blocked by EA, intracellular resistin protein was not accumulated due to increased protein degradation compared to *de novo* synthesis in the adipocytes. Moreover, the effect of EA on resistin secretion was not accompanied by the transcriptional change of resistin. Yang et al. reported that EA inhibits the fusion

of secretory vesicles with their target membranes by intercalating soluble *N*-ethylmaleimide-sensitive factor attachment complex [29]. This unique property of EA could also inhibit resistin secretion by affecting the specific vesicular machinery involved. In KK-A^y mice, EA also reduced serum resistin levels without affecting resistin mRNA expression in the adipose tissue (Fig. 2A, B), which suggests that the inhibitory effect of EA on serum resistin levels could be caused by the property described above.

EA improved not only serum resistin levels (Fig. 2A) but also hepatic steatosis and serum lipid composition in obese KK-A^y mice (Table 1). One of the target sites of resistin is known to be liver. Resistin knockout mice exhibited reduced hepatic glucose production due to activation of adenosine monophosphate-activated protein kinase and decreased expression of gluconeogenic enzymes in the liver [15]. Gene ablation of resistin in leptin-deficient mice improved hyperlipidemia and hepatic steatosis [18]. In HepG2, a human hepatic cell line, exposure to resistin at the concentration observed in obese individuals increased the protein expression of apolipoprotein B and cellular lipid content [30], and suppressed the protein expression of LDL receptor [31]. Furthermore, both the injection of resistin in mice and incubation of HepG2 cells with resistin, reduced mitochondrial content in the liver [32] which is known to occur in the metabolic syndrome [33]. In our study, EA supplementation improved hepatic steatosis, reduced TG content in the liver (Fig. 1), and ameliorated serum HDL-C and non-HDL-C levels (Table 1), which might be caused by reduced serum resistin levels (Fig. 2A) and partly by transcriptional changes of *apoa1* and *ldlr* in the liver (Fig. 3A). Although it remains unclear how resistin affects the liver functions, our data suggested that EA could indirectly affect liver functions through the regulation of serum resistin levels.

Measurements of several gene expressions in the liver with quantitative RT-PCR showed that EA supplementation altered mRNA expression of genes involved in fatty acid degradation compared to that in control mice (Fig. 3C). EA supplementation significantly upregulated mRNA expressions of *cpt1a* and *ppara* in the liver compared with that in control mice (Fig. 3C). PPARα is a master regulator of fatty acid oxidation through transcriptional control of its target genes including *cpt1a*, and the presence of its activator in diet fed to KK-A^y mice reduced plasma and hepatic TG [34] as same as that seen in our present study, suggesting that EA acts as transcriptional activator of PPARα in the liver. To our knowledge, it is not clear that reduced resistin levels in serum leads to transcriptional activation of PPARα in the liver. Moreover, gene ablation of resistin in leptin knockout mice reduced mRNA expressions of *srebf1c*, *acaca*, and *fasn*, which were upregulated in leptin knockout control mice. However, in our study, mRNA expressions of *srebf1c*, *acaca*, and *fasn* in the liver of EA group were not changed or upregulated compared with that of control group (Fig. 3B). Therefore, transcriptional up-regulation of these genes in the liver is independent of reduced serum resistin levels but is a specific effect of EA supplementation.

In conclusion, our study indicated that EA not only suppresses resistin secretion *in vivo*, but also improves hepatic functions, hepatic steatosis, and serum lipid profile in obese type 2 diabetic KK-A^y mice. These findings suggested that EA is a plant food-derived functional component, which can improve pathologies in metabolic syndrome such as hyperlipidemia and type 2 diabetes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.100.

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