Dietary Corosolic Acid Ameliorates Obesity and Hepatic Steatosis in KK-Ay Mice

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Corosolic acid (CRA), a constituent of Banaba leaves, has been reported to exert anti-hypertension, anti-hyperinsulinemia, anti-hyperglycemia, and anti-hyperlipidemia effects as well as to induce anti-inflammatory and anti-oxidative activities. The aim of this study was to investigate the inhibitory effects of CRA on the development of obesity and hepatic steatosis in KK-Ay mice, a genetically obese mouse model. Six-week-old KK-Ay mice were fed a high fat diet for 9 weeks with or without 0.023% CRA. Nine-week CRA treatment resulted in 10% lower body weight and 15% lower total fat (visceral plus subcutaneous fat) mass than in control mice. CRA treatment reduced fasting plasma levels of glucose, insulin, and triglyceride by 23%, 41%, and 22%, respectively. The improved insulin sensitivity in CRA-treated mice may be due on part to the increased plasma adiponectin and white adipose tissue (WAT) AdipoR1 levels. In addition, CRA treatment increased the expression of peroxisome proliferator-activated receptor (PPAR)α in liver and PPARγ in WAT. This is the first study to show that CRA treatment can contribute to reduced body weight and amelioration of hepatic steatosis in mice fed a high fat diet, due in part to increased expression of PPARα in liver and PPARγ in WAT.

Key words: corosolic acid; peroxisome proliferator-activated receptor; obesity; hepatic steatosis

Banaba leaves (Lagerstroemia speciosa LINN.) have been used as a traditional medicine in Southeast Asia, and tea made from the leaves is used as a treatment for diabetes. The leaves contain large amounts of corosolic acid (CRA) (Fig. 1), which recently has attracted attention for its biological properties.1–7 It was shown that Banaba leaf extract (1% CRA) treatment for 2 weeks decreased blood glucose levels in humans.8 In addition, CRA (10 mg) has been shown to reduce post challenge plasma glucose levels at 90 min in humans.7 We previously reported that CRA ameliorates hypertension and abnormal lipid metabolism as well as mitigating oxidative stress and the inflammatory state in SHR/NIdmcr-cp rats on a high fat diet,9 that is an animal model of metabolic syndrome.10 We also found that the administration of CRA (10 mg/kg) in a normal diet for 2 weeks improved hyperglycemia by reducing insulin resistance in a KK-Ay mouse, an obese animal model that spontaneously develops hypertriglyceridemia, hyperglycemia, hyperinsulinemia, and diabetes.11,12 However, the effects of CRA on the adipokines and transcription factors that play central roles in the development of obesity are unknown.

Obesity and type 2 diabetes in both humans and animals are associated with insulin resistance.13 Adiponectin is an insulin-sensitizing hormone, and plasma levels of adiponectin have been reported to be significantly reduced in obese/diabetic mice and humans.14–16 It also was reported that adiponectin receptors, AdipoR1 and AdipoR2, are downregulated in adipose tissue and skeletal muscle in obese diabetic ob/ob mouse, which is correlated with decreased adiponectin sensitivity.17

Abnormalities of lipid metabolism are frequently observed in obesity and type 2 diabetes. Peroxsome proliferator-activated receptor (PPAR)α is predominantly expressed in liver, and regulates the expression of genes involved in lipid metabolism. Activators of PPARα decrease circulating lipid levels and are commonly used to treat hypertriglyceridemia and other dyslipidemic states. Recent studies suggest that the activation of PPARα prevents high fat diet-induced obesity,18 insulin resistance,18,19 and hepatic steatosis.20 It was also found that activation of PPARα increased AdipoR1 and AdipoR2 expression in adipocytes in vivo.21

Growth and differentiation of adipocytes are regulated by PPARγ, which is highly expressed in adipose tissue. Activation of PPARγ by agonists such as thiazolidinediones increases the number of small adipocytes, which increases the amount of the insulin-sensitizing hormone adiponectin.15,22 PPARγ activation also stimulates lipid storage in adipocytes and reduces lipotoxicity in liver and skeletal muscle, thereby improving insulin sensitivity.23

In the present study, we examined the inhibitory effects of CRA on the development of obesity and hepatic steatosis in

Fig. 1. Structure of Corosolic Acid (CRA)
KK-Ay mice using 43 mg/kg CRA, a dosage similar to that used in the previous study. We performed biochemical measurements, body composition analysis, and morphological analysis of liver and white adipose tissue (WAT). In addition, we examined gene and protein expression of PPARα in liver, which regulates β-oxidation of fatty acid. We also examined gene expression of AdipoR1 and AdipoR2, which are increased by PPARα agonist, and PPARα, which increases adiponectin, in WAT.

MATERIALS AND METHODS

Chemicals CRA provided by Use Techno Corporation (Kyoto, Japan) was stored at room temperature until use. All other chemicals were of reagent grade.

Animals Male KK-Ay mice were purchased from Clea Japan (Tokyo, Japan). The mice were housed in an air-controlled room (temperature 25±2 °C and 50% humidity) with a 12 h light/dark-cycle and food and water provided ad libitum. Six-week-old male KK-Ay mice (1 mouse/cage) were fed a high fat diet with (CRA-treated mice) or without (control mice) 0.023% (w/w) CRA for 9 weeks. The high fat diet contained 45% kcal as fat, 35% kcal as carbohydrate, and 20% kcal as protein, with an energy density of 3.57 kcal/g. All studies were performed in the laboratories of the Department of Diabetes and Clinical Nutrition, Kyoto University, in accordance with the Declaration of Helsinki. The Animal Care Committee of Kyoto University Graduate School of Medicine approved animal care and procedures.

Computed Tomography (CT)-Based Body Composition Analysis CT-based analysis of body composition was performed after 9 weeks. The mice were anesthetized with intraperitoneal injections of pentobarbital sodium, and scanned using a LaTheta (LCT-100M) experimental animal CT system (Aloka, Tokyo, Japan). Contiguous 1 mm slice images of the whole abdominal cavity (Fig. 3A) were used for quantitative assessment by La Theta software (version 1.00), as described previously. Visceral plus subcutaneous fat mass (total fat mass) and lean body mass were evaluated quantitatively.

Histological Examination Epididymal white adipose tissue (WAT) after 9 weeks was removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. The diameter of the adipocytes was measured by microscopy (BZ-8000, Keyence, Osaka, Japan); 50 cells per mouse were measured in several parts of the epididymal fat pad. Liver was fixed in 4% paraformaldehyde in 0.1 m phosphate-buffered saline for 2 h at 4 °C, frozen, sectioned, stained with Oil Red O, and counterstained with hematoxylin.

Measurement of Liver Triglyceride (TG) Content To analyze hepatic TG levels, approximately 0.2 g of tissue was homogenized and extracted in a chloroform : methanol mixture (2:1 v/v). Triglyceride was enzymatically quantified using a TG E Test Wako (Wako Pure Chemical Industries, Osaka, Japan).

Biochemical Measurements Blood samples were collected in heparinized capillary tubes and centrifuged at 2400 g. Plasma TG and glucose levels were measured using a TG E test and Glucose II test, respectively (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin levels were measured using a mouse insulin ELISA kit (Shibayagi, Gunma, Japan). Plasma adiponectin levels were measured by mouse/rat adiponectin immunoassay kit (Otsuka Pharmaceutical, Tokushima, Japan).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was isolated from liver and fat with Trizol reagent (Invitrogen, CA, U.S.A.), and examined by real-time quantitative RT-PCR using a PE Applied Biosystems prism model 7000 sequence detection instrument. SYBER Green PCR Master Mix (Applied Biosystem, CA, U.S.A.) was prepared for the PCR run. Thermal cycling conditions were denaturation at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The sequences of the sense and antisense primers of PPARα, PPARγ, AdipoR1 and AdipoR2 used for amplification from mouse liver and WAT were as follows: the sense primer for PPARα was cctgtagctggttgctgaa, the antisense primer tgcagctgcataccatgt; the sense primer for PPARα was tgcggtctgagatacct, the antisense primer gctgacagatcagcagactct; the sense primer for AdipoR1 was 5'-acctggagagctcctct-3', the antisense primer 5'-ctctggtgatgcgaagagat-3'; the sense primer for AdipoR2 was 5'-ccaggaagatgagggctttat-3', the antisense primer 5'-tcactgtgatcagatgata-3'. Gene expression levels were corrected for GAPDH mRNA level.

Protein Analysis For PPARα analysis, nuclear extracts were prepared essentially as described by Gebel et al. Briefly, frozen liver samples (0.1 g) were homogenized in 0.5 ml of ice cold buffer containing 20 mM Tris–HCl (pH 7.4), 10% glycerol, 1 mM EDTA, 1% dithiothreitol together with protease inhibitors (Complete, EDTA Free; Roche, Mannheim, Germany), and the nuclei were then pelleted and resuspended in fresh buffer 0.4 M NaCl. The suspensions were mixed (4 °C, 30 min) and centrifuged (2000×g, 4 °C, 30 min), and supernatants (nuclear extracts) were collected. Protein was analyzed using a kit from Bio-Rad Laboratories (CA, U.S.A.) with bovine serum albumin as standard.

Nuclear Extracts (15 μg protein) were 12% SDS-polyacrylamide gels, transferred to PVDF membranes, which were blocked in TBST buffer (20 mM Tris–HCl [pH 7.5] and 55 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk powder and incubated with primary antibody 24 h. Primary antibodies and dilutions used were as follows: mouse anti-GAPDH (1:5000, Chemicon, CA, U.S.A.); rabbit anti-murine PPARα (1:500, Santa Cruz Biotechnology, SantaCruz, CA, U.S.A.). TBST was used as wash buffer and antibody diluent. After washing for 3×10 min, blots were incubated (60 min) with horseradish peroxidase-conjugated secondary anti-bodies, either anti-rabbit IgG (1:5000, GE Healthcare, Buckinghamshire, U.K.) or anti-mouse IgG (1:5000, GE Healthcare, Buckinghamshire, U.K.). Blots were given final washes (2×15 min) and antibody binding was detected on X-ray film by enhanced chemiluminescence (ECL Advance, GE Healthcare, Buckinghamshire, U.K.).

Statistics Results are expressed as mean±S.E.M. Statistical analysis was performed using unpaired Student’s t-test for data from control and CRA-treated mice. Differences was considered significant at p<0.05.
RESULTS

Effects of CRA on Body Weight and Fat Mass in KK-Ay Mice

KK-Ay mice were fed a high fat diet with (CRA-treated mice) or without (control mice) 0.023% (w/w) CRA for 9 weeks. As shown in Fig. 2, mice treated with CRA for 9 weeks had 10% less body weight than control mice. In normal diet-fed mice, there were no significant differences in CRA-treated or control mice (data not shown). There were no significant differences in food intake between CRA-treated (460±10 g/9 weeks) and control mice (454±8 g/9 weeks). As shown in Fig. 3, a significant decrease in total fat mass (Fig. 3B, 15%, p<0.05) and visceral fat mass (Fig. 3D, 16%, p<0.05) was observed in CRA-treated mice compared to controls. Subcutaneous fat mass was somewhat decreased, but not significantly, in the CRA-treated mice (Fig. 3C, 14%, p<0.09). Lean body mass was similar in control and CRA-treated mice (Fig. 3E).

Effects of CRA on Fasting Plasma Glucose, Insulin, TG and Fed Plasma Adiponectin Levels

As shown in Fig. 4, the CRA-treated mice showed levels of fasting plasma glucose (A) and insulin (B) reduced by 23% (p<0.05) and 41% (p<0.05), respectively, compared to controls, suggesting that the CRA-treated mice were more insulin sensitive. CRA-treated mice showed fasting plasma TG (Fig. 4C) levels reduced by 22% (p<0.05) compared to controls. Fed plasma levels of adiponectin were higher (16%, p<0.05) in CRA-treated mice compared to controls (Fig. 4D).

Histology of Epididymal WAT and Effects of CRA on WAT mRNA Expression Levels of AdipoR1, AdipoR2 and PPARγ

Figure 5A shows representative sections of epididymal WAT from control and mice treated with CRA for 9 weeks, stained with hematoxylin and eosin. As shown in Fig. 5B, the size of adipocytes of epididymal WAT of CRA-treated mice was significantly less (28%, p<0.05) than that of controls. AdipoR1 mRNA expression levels were significantly higher in CRA-treated mice (15%, p<0.05) than in controls (Fig. 5C), although there was no difference in AdipoR2 mRNA between control and CRA-treated mice (Fig. 5D). CRA-treated mice showed significantly elevated PPARγ mRNA expression levels (60%, p<0.05) compared to controls (Fig. 5E).

Histology of Liver, Liver TG Content, and Hepatic mRNA and Protein Expression of PPARγ

Figure 6A shows Oil Red O-stained sections of liver of control mice and CRA-treated mice. The liver of control mice showed widespread deposition of fat globules of various sizes. In contrast, the liver of CRA-treated mice showed less fat accumulation, indicating less hepatic steatosis than in controls.
The liver weight of CRA-treated mice was significantly less (35%, \( p < 0.05 \)) than in controls (Fig. 6B), which is associated with the significantly decreased hepatic TG accumulation in CRA-treated mice (37%, \( p < 0.05 \)) (Fig. 6C). Since PPAR\( \alpha \) plays an important role in maintaining homeostasis of lipid metabolism, PPAR\( \alpha \) mRNA expression was examined in liver samples from controls and mice treated with CRA for 9 weeks. As shown in Fig. 6D, expression of PPAR\( \alpha \) mRNA in CRA-treated mice was significantly higher (64%, \( p < 0.05 \)) than in controls. We also confirmed protein expression of PPAR\( \alpha \) and found that CRA-treated mice showed significantly higher (31%, \( p < 0.05 \)) PPAR\( \alpha \) protein levels than in controls (Fig. 6E).

**DISCUSSION**

This is the first study to show that CRA treatment in mice fed a high fat diet contributes to reduced body weight and hepatic steatosis associated with increased expression of PPAR\( \alpha \) in liver and PPAR\( \gamma \) in WAT. CRA treatment also may improve insulin sensitivity by increasing plasma adiponectin and WAT AdipoR1 levels.

In the present study, we found that CRA increased mRNA and protein expression of PPAR\( \alpha \) in liver, suggesting that CRA may act as a PPAR\( \alpha \) agonist. The decreased body weight and fat mass observed in the CRA-treated mice are most likely due to increased \( \beta \)-oxidation and energy expenditure by PPAR\( \alpha \) activation, as seen in mice treated with PPAR\( \alpha \) agonists. Hypertriglyceremia and hepatic steatosis are also ameliorated by CRA treatment, in a manner similar to that by PPAR\( \alpha \) agonists, by increasing fatty acid \( \beta \)-oxidation in liver. CRA-treated mice on a high fat diet showed significantly reduced liver weight compared to controls, due to the significantly decreased hepatic TG accumulation. However, there are several reports of increased liver weight by synthetic PPAR\( \alpha \) agonists in rodents on a normal diet through induction of DNA synthesis and suppression of apoptosis in hepatocytes. In the present study, increased AdipoR1 expression in WAT was found in the CRA-treated mice, which may well contribute partly to the CRA-related improvement in insulin sensitivity.

In addition, CRA treatment was found in the present study...
to increase PPARγ expression in WAT. Treatment of obese diabetic mice with thiazolidinediones, a PPARγ agonist, has been suggested to stimulate adipocyte differentiation and apoptosis, thereby preventing adipocyte hypertrophy, which is consistent with our results. Increased plasma adiponectin levels also play an essential role in improved insulin sensitivity in CRA-treated mice, most likely due to increased number of small adipocytes through CRA-induced activation of PPARγ in WAT, resulting in decreased plasma glucose levels.

It is noteworthy that CRA can activate both PPARα and PPARγ. Tsuchida et al. reported that activation of PPARα and PPARγ by combination therapy of Wy-14643 and rosiglitazone improved insulin sensitivity and reduced body weight in KK-Ay mice.31 Compounds that have dual agonistic activity on both of these receptors have been shown to exhibit several beneficial metabolic effects in obese diabetic animals. Ragaglitazar, a PPARα and PPARγ agonist, was shown to reduce plasma TG and glucose levels in high fat animals. Ragaglitazar, a PPARα and PPARγ agonist, has been suggested to stimulate adipocyte differentiation and PPARγ expression in liver and CRA-induced adiponectin levels also play an essential role in improved insulin sensitivity.

In conclusion, we have demonstrated that CRA ameliorates high fat diet-induced obesity and hepatic steatosis in genetical obese KK-Ay mice, at least in part, by increasing PPARγ expression in liver and PPARγ expression in WAT. CRA treatment also improved insulin sensitivity by increasing plasma adiponectin levels and WAT AdipoR1 levels. As obese patients are likely to prefer a high fat diet, CRA may be an effective therapeutic option for the treatment of obesity and diabetes.

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