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ORIGINAL ARTICLE

Metabolomic analysis of amino acid and energy metabolism in rats supplemented with chlorogenic acid

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Abstract This study was conducted to investigate effects of chlorogenic acid (CGA) supplementation on serum and hepatic metabolomes in rats. Rats received daily intragastric administration of either CGA (60 mg/kg body weight) or distilled water (control) for 4 weeks. Growth performance, serum biochemical profiles, and hepatic morphology were measured. Additionally, serum and liver tissue extracts were analyzed for metabolomes by high-resolution ^{1}H nuclear magnetic resonance-based metabolomics and multivariate statistics. CGA did not affect rat growth performance, serum biochemical profiles, or hepatic morphology. However, supplementation with CGA decreased serum concentrations of lactate, pyruvate, succinate, citrate, β-hydroxybutyrate and acetoacetate, while increasing serum concentrations of glycine and hepatic concentrations of glutathione. These results suggest that CGA supplementation results in perturbation of energy and amino acid metabolism in rats. We suggest that glycine and glutathione in serum may be useful biomarkers for biological properties of CGA on nitrogen metabolism in vivo.

Keywords Chlorogenic acid · Amino acids · Metabolism · Nuclear magnetic resonance spectroscopy

Abbreviations

CGA	Chlorogenic acid
PC	Principal components
PCA	Principal component analysis
NMR	Nuclear magnetic resonance

Introduction

Phenolic acids are currently receiving much attention because of their beneficial health effects related to their anti-oxidant, anti-inflammatory and anti-cancer properties (Cai et al. 2004; Trouillas et al. 2003). Chlorogenic acid (CGA) is one of the most abundant hydroxy cinnamic acid (phenolic acids) compounds in the human diet, such as coffee and tea (Algamdi et al. 2011; Meng et al. 2013). Accumulating evidence shows that CGA exhibits many biological effects, including anti-bacterial, anti-oxidant, anti-inflammation, anti-cancer, anti-diabetes, anti-hypertension and anti-obesity actions (Greenberg et al. 2006; Kasai et al. 2000; Lou et al. 2011; Yun et al. 2012; Zhao et al. 2012). There are also reports that CGA modulates glucose and lipid metabolism both in healthy and genetically metabolic disordered conditions (Huang and Chang

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2008; Li et al. 2009; Zhang et al. 2011). Furthermore, CGA confers hepato-protective benefits on protecting animals from LPS-induced or chemical injury (Shi et al. 2012; Yun et al. 2012). For example, intraperitoneal administration of CGA (50 mg/kg body weight) suppresses hepatic mRNA levels for toll-like receptor 4 (TLR4), TNF-alpha and NF-kappa B p65 subunit, thereby protecting the liver from LPS-induced injury (Xu et al. 2010). Furthermore, intraperitoneal administration of CGA to CCl4-intoxicated mice (100 mg/kg body weight per day) for 8 days ameliorates lipid peroxidation, hepatic injury, cytochrome P450 (Cyt P450) inactivation, leading to the enhancement of the cellular anti-oxidant defense (Marques and Farah 2009). At present, little is known about effects of oral administration of CGA on anti-oxidant activity or nutrient metabolism in vivo. Wan et al. (2013) found that dietary supplementation with CGA (1 or 10 mg/kg body weight/day) attenuated both the elevated plasma concentrations of total cholesterol and low-density lipoprotein and the decreased plasma concentrations of high-density lipoprotein in rats fed a hypercholesterolemic diet. The hypocholesterolemic effect of CGA may result from altered metabolism of nutrients, including fatty acids, glucose and amino acids.

Previous studies of the biological effects of CGA have been confined only to measurements of a single or several biochemical markers in tissues (Shi et al. 2012; Yun et al. 2012). This approach does not adequately reflect the overall metabolism of nutrients in animals or humans. These parameters are commonly preselected based on a hypothesis. Therefore, the traditional methods cannot be used to perform global screening of physiological perturbations, such that unexpected or novel responses or biomarkers are often not detected. Hence, it is necessary to employ a robust technique to simultaneously quantify and identify a large number (hundreds to thousands) of molecules in blood and other tissues (He et al. 2012; Liu et al. 2011).

Metabolomics has emerged as a biomarker discovery tool for metabolic profiling in nutritional research (He et al. 2011; Lindon et al. 2001). Nuclear magnetic resonance (NMR) spectroscopy is one of the major techniques used in metabolomic studies as the spectra of biofluids or tissues contain a wealth of metabolic information and provide comprehensive biochemical profiles of low-molecular-weight metabolites whose concentrations are precisely regulated in response to various stimuli (Wang et al. 2009). Thus, NMR analysis aids in enhanced understanding of molecular mechanisms and in providing novel insight into the intervention effect or perturbation of diets with regard to nutrient metabolism and health. To capitalize on this advanced technique, the present study was conducted with ^{1}H NMR to screen the global metabolic profile of the serum and liver of CGA-supplemented rats.

Table 1 Composition of the experiment diet

Ingredients	Content (%)	Chemical composition	Content
Wheat	14	Digestible energy (Mcal/kg)	3.40
Corn	43	Crude protein (%)	21.0
Soybean meal	24	Crude fat (%)	4.5
Full fat soybean extruded	8	Dry matter	90.0
Soybean oil	1.4	Calcium (%)	1.0
Whey powder	3	Total phosphate (%)	0.7
Fish meal	3.2	Sodium (%)	0.3
Limestone	1.3	Met + Cys (%)	0.78
Dicalcium phosphate	1.1	Lys (%)	1.35
Vitamin-mineral premix[a]	1.0	Thr (%)	0.88

The nutrient levels of the diet were based on China General Quality Standards for Animal Feed (GB14924.1-2001)

[a] The vitamin-mineral premix provided (per kilogram feed): vitamin A, 14,000 IU; vitamin D_3, 1,500 IU; vitamin E, 5 mg; vitamin K, 5 mg; thiamine, 13 mg; riboflavin, 12 mg; pyridoxine, 12 mg; vitamin B12, 0.022 mg; niacin 60 mg; pantothenic acid, 24 mg; biotin, 0.2 mg; folic acid, 6 mg; choline chloride, 350 mg; Fe (as iron sulfate), 120 mg; Cu (as copper oxide), 10 mg; Mn (as manganous oxide), 75 mg; Zn (as zinc oxide), 30 mg; I (as ethylenediamine dihydroiodide), 0.5 mg; and Se (as sodium selenite), 0.2 mg

Materials and methods

Dietary treatment and sample collection

This study was approved by the Nanchang University Animal Experiment Ethics Committee, carried out in the Center for Disease Control and Prevention of Jiangxi Province (Nanchang, China), and performed in accordance with the Chinese guidelines for the Laboratory Animals Care. A total of 12 adult female Sprague–Dawley rats (weighting 170–190 g) were obtained from Changsha Tianqin Biotechnology Co., Ltd (Changsha, China). They were housed individually in metabolism cages after acclimation for 7 days in plastic cages, and had free access to the standard rodent diet and drinking water. The animal room was well ventilated and maintained at a cycle of 12 h light and 12 h darkness, 22–25 °C, and the relative humidity of 50 % throughout the study. The experimental diets (Table 1) were formulated to meet nutrient requirements for rats (Xu et al. 2013).

Rats were randomly divided into two groups: the control group (oral administration of distilled water once daily) and the CGA group (oral administration of CGA once daily). CGA with a purity of 98 % (Aladdin Co., Ltd, Shanghai, China) was dissolved in distilled water and orally administered at a dose of 60 mg/kg body weight. This dosage of CGA was based on previous reports (Shi et al. 2009, 2012).

Water or CGA was given daily to a respective treatment group for 28 days. After the last administration of water or CGA, rats were euthanized (Ruan et al. 2013) following a 12-h period of food deprivation to avoid a postprandial effect on serum metabolites. Liver (~5 g) and blood samples (~5 mL) were quickly collected. Sera were separated from whole blood by centrifugation at 600×*g* and 4 °C for 15 min (Ren et al. 2013a). All serum and liver samples were stored at −80 °C until analysis.

Growth performance

The body weight of each rat was recorded every day. The daily weight gain and food intake of the rats were measured. Feed conversion ratio (FCR) was calculated based on the average daily feed intake of rats divided by their body-weight gain (Foo et al. 2003; He et al. 2003).

Serum biochemical parameters

Serum metabolites were determined using a biochemistry analyzer (Beckman, USA) according to the manufacturer's protocol (Nanjing Jiancheng Technology Co., Ltd, Nanjing, China), as previously described (Tan et al. 2012; Ren et al. 2013b). They included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, glucose (GLU), lactate dehydrogenase (LDH), albumin, total protein, cholesterol, triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL).

Liver index and histological analysis

Liver index was calculated by the following equation: Liver index (%) = (liver weight/body weight) × 100 (Mahaffey et al. 1981). Hematoxylin and eosin staining and histological analysis were performed as previously reported (He et al. 2013; Yao et al. 2011). Liver tissue was fixed by immersion in 10 % neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5 μm thickness, affixed to slides, deparaffinized, stained with hematoxylin and eosin, and examined by light microscopy. Photomicrographs were acquired with 200× magnifications using an Olympus BX51 microscope (Olympus Optical Company, Shanghai, China).

Sample preparation and ^{1}H NMR measurement

Serum samples were prepared by mixing 350 μL serum and 350 μL D_2O and through centrifugation at 10,000×*g* for 10 min. The supernatant fluid (550 μL) was collected and transferred into 5-mm NMR tubes. All ^{1}H NMR spectra were acquired on a Bruker Advance DRX-600 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a proton frequency of 600.58 MHz and a temperature of 294 K, using a cryogenic high-resolution probe. ^{1}H NMR spectra of serum samples were acquired using the Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence to emphasize resonances from low-molecular-weight metabolites. For each sample, a total of 64 transients were collected into 45-K data points over a spectral width of 9615.4 Hz with a relaxation delay of 4.0 s and an acquisition time of 3.4 s (Ruan et al. 2013).

A pre-weighted liver tissue (0.1 g) was homogenized in 300 μL buffer solution (0.2 mol/L Na_2HPO_4 and 0.2 mol/L NaH_2PO_4, pH 7.4) and 400 μL methanol-d (CH_3OD). The samples were allowed to stand for 20 min prior to centrifugation at 10,000×*g* at 4 °C for 15 min to remove any precipitates. Aliquots of the supernatant fluid (550 μL) from each sample were transferred into a 5-mm NMR tube containing 30 μL of 2 mmol/L TMSP [3-(trimethylsilyl) propionic-(2,2,3,3-d4) acid sodium salt] (TMSP dissolved in D_2O). Aqueous liver extracts were analyzed using NOESYGPPR-1D pulse sequence (relaxation delay-90°-t_1-90°-t_m-90°-acquisition) with solvent presaturation (Schnackenberg et al. 2005; Yang et al. 2007). For each sample, a total of 64 transients were collected into 32-K data points over a spectral width of 9,615.4 Hz with a relaxation delay of 4.0 s and an acquisition time of 3.4 s.

Data processing and statistical analysis

All ^{1}H NMR spectrums from serum and liver extracts were phase-adjusted and baseline-corrected with MNova-6.1.1 (MestreLab, Santiago de Compostela, Spain). For serum samples, NMR spectra were referenced to the lactate-CH_3 signal at 1.33 ppm. The TMSP acted as a chemical shift reference (δ0.0) in spectrum from liver extracts. Each spectrum over the range of δ0.5–9.0 was divided into integrated regions of equal width (0.01 ppm). The region of δ4.60–5.10 and δ4.70–5.20, which included the residual water resonance, was removed from all liver and serum spectra, respectively. In order to avoid any baseline distortion caused by imperfect water suppression, the region of residual methanol δ3.29–3.35 was removed from all liver spectra. Each integral region was then normalized to the sum of total integral regions. The data were imported into Microsoft Excel (version 2003) and included in SIMCA-P 12.0 as variables. The resulting data of principal component analysis (PCA) were demonstrated by the PCA scores' plots and loadings' plots (Wu 2010). Score plots of the first two principal components (PCs) were used to visualize the separation of two groups, and loading plots reflected the NMR spectra regions that were altered as a result of the CGA treatment. The normalized integral values were then input into the software of SPSS 17.0 (SPSS Inc., Chicago, USA) for statistical analysis. The

Table 2 Effects of CGA on growth performance in rats

Group	Initial body weight (g)	Finish body weight (g)	ADG (g/day)	ADFI (g/day)	FCR (g/g)
Control	179.00 ± 3.19	255.80 ± 3.69	2.74 ± 0.10	18.50 ± 1.09	6.74 ± 0.23
CGA	187.00 ± 3.10	265.33 ± 6.58	2.80 ± 0.24	19.59 ± 0.62	7.01 ± 0.37

Values are mean ± SEM, $n = 6$

ADG average daily gain, *ADFI* average daily feed intake, *FCR* feed conversion rate (average daily feed intake/body weight gain)

Table 3 Effects of CGA on serum biochemical profiles in rats

Items	Control group	CGA group
ALT (IU/L)	73.0 ± 3.4	74.0 ± 0.9
AST (IU/L)	175 ± 7.3	184 ± 2.5
ALP (U/L)	115 ± 13	111 ± 4.4
TBIL (mg/L)	0.50 ± 0.03	0.52 ± 0.04
Glucose (mmol/L)	4.68 ± 0.20	4.63 ± 0.26
LDH (U/L)	1502 ± 62	1418 ± 57
Albumin (g/L)	20.5 ± 0.9	22.6 ± 0.5
Total protein (g/L)	79.4 ± 1.7	81.3 ± 2.2
Cholesterol (mmol/L)	2.14 ± 0.09	2.30 ± 0.17
Triglyceride (mmol/L)	0.91 ± 0.07	1.01 ± 0.06
LDL (mmol/L)	0.20 ± 0.02	0.18 ± 0.01
HDL (mmol/L)	2.02 ± 0.06	2.21 ± 0.13

Values are mean ± SEM, $n = 6$

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *TBIL* total bilirubin, *LDH* lactate dehydrogenase, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein

independent-sample t test method was used to detect significant differences in signals between two groups (Fu et al. 2010). Probability values ≤ 0.05 were taken to indicate statistical significance (Wei et al. 2012).

Results

General observations

The growth performance of rats is shown in Table 2. Final body weight, ADG, ADFI or FCR did not differ ($P > 0.05$) among all the treatment groups. CGA supplementation had no effect on the serum biochemical profiles of rats, as measured using the conventional methods (Table 3). Overall, CGA supplementation did not affect the liver index or morphology, as compared with the control group (Fig. 1).

^{1}H NMR spectrum of serum and liver

Typical ^{1}H NMR spectra of two serum samples taken from the control and CGA groups are illustrated in Fig. 2a and b, respectively. Similarly, typical ^{1}H NMR spectra of aqueous extracts of two liver samples taken from the control and CGA group rat are shown in Fig. 2c and d, respectively. A number of perturbations in endogenous metabolites are revealed by the ^{1}H NMR spectra. A total of 35 metabolites in the serum and 36 metabolites in the liver were unambiguously assigned for the ^{1}H NMR spectra. Assignment of metabolites was made on the basis of comparisons with the published literature (Jiang et al. 2013; Liao et al. 2007; Zira et al. 2013). Their chemical shifts and peak multiplicity are listed in Tables 4 and 5, respectively.

To detect more subtle treatment-related metabolic differences, pattern recognition techniques were applied. PCA was performed on the ^{1}H NMR spectra of serum and liver samples in the control and CGA groups. The PCA scores plot of the ^{1}H NMR serum data are summarized in Fig. 3a. This plot shows the first two PCs and accounts for 83.6 % of the variation in the samples. A PCA score plot of the ^{1}H NMR from liver samples is given in Fig. 3c. This plot displays the first two PCs and explains 59.8 % of the total variations within the data. Figure 3a and c intuitively illustrate the discrete trend of two sets of the data.

Each triangle in the loading plots (Fig. 3b, d) represents a single NMR spectral region or chemical shifts. The loading plots indicated the spectral variables of metabolites. The distance between triangle and origin in the loading plots represented the contribution of the corresponding metabolite to differences between the two groups of rats. The corresponding loading plot (Fig. 3b) showed increases in ^{1}H NMR peaks for lipoproteins, glycine and unsaturated lipids in the serum of CGA-supplemented rats and decreases in ^{1}H NMR peaks for β-hydroxybutyrate, lactate, acetoacetate, pyruvate, succinate and citrate (Table 6). They are the major contributors to the separate clustering of the groups. Detailed analysis of the loadings plot from Fig. 3d indicated that the level of hepatic glutathione was increased ($P < 0.05$) in the CGA group, as compared with the control group (Table 7).

Discussion

There is growing interest in phytochemicals as dietary supplements for humans and animals (Kong et al. 2011). In the current work, we sought to assess the effects of

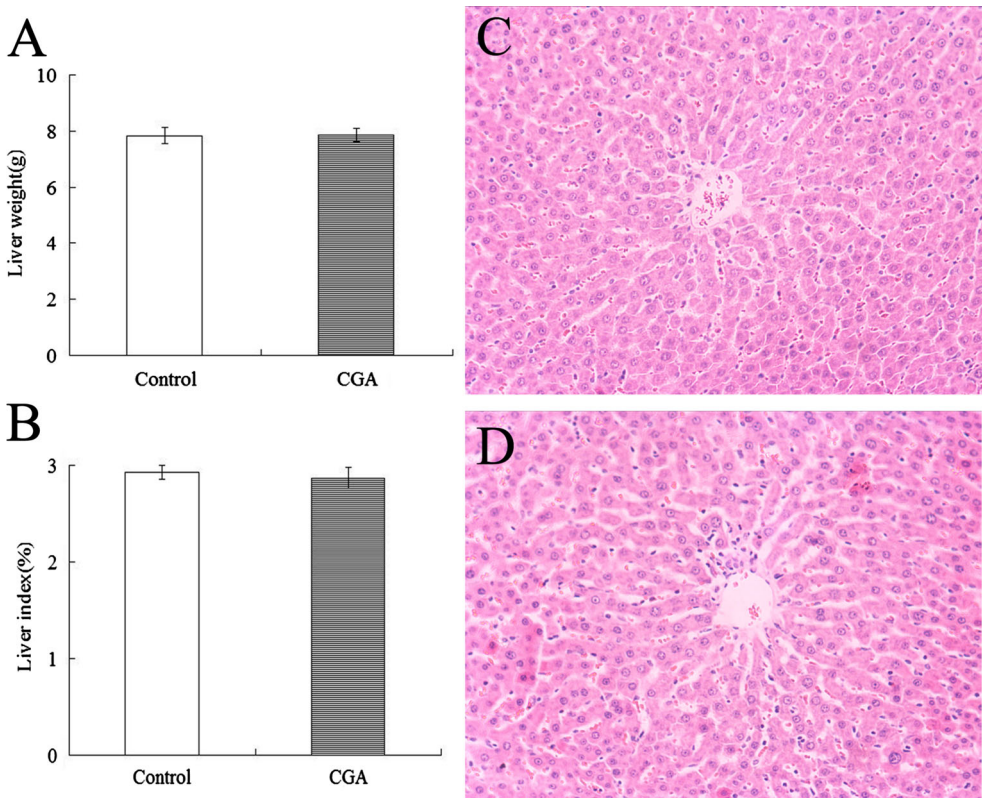

Fig. 1 **a**, **b** Effects of CGA on liver weight and liver index of rats in the control group and CGA group. Values are mean ± SEM, $n = 6$. **c**, **d** Histological features of liver sections stained with H&E, where typical images were chosen from the control group and CGA group (original magnification ×200); **c** the liver of rats in the control group; **d** the liver of rats in the CGA group

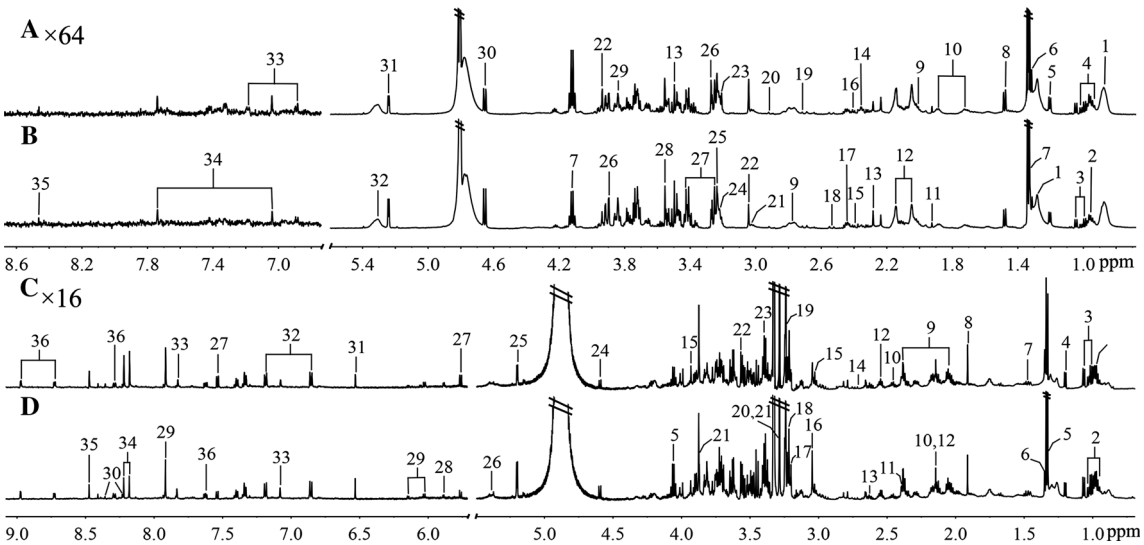

Fig. 2 Typical ^{1}H NMR spectra (600 MHz) of serum obtained from rats in the control group (*A*) and the CGA group (*B*), as well as aqueous soluble liver extracts obtained from rats in the control group (*C*) and the CGA group (*D*)

CGA supplementation on serum metabolic profiles and hepatic metabolism in rats. Our results support the hypothesis that CGA supplementation can regulate nutrient metabolism and enhance the anti-oxidant function in animals, without negatively affecting growth performance, serum lipid profiles or liver morphology in rats (Tables 2, 3; Fig. 1). Of note, several studies reported that CGA supplementation could protect the liver against stress or pathologic condition-induced injury (Shi et al. 2012; Yun et al. 2012). Similarly, CGA present in coffee is capable of quenching radical species and inhibiting H_2O_2-induced apoptosis via suppressing mitochondrial membrane depolarization and caspase-9 activation in cells (Park 2013). Furthermore, CGA

Table 4 Relative changes in concentrations of serum metabolites in CGA-supplemented rats on the basis of the chemical shift of the methyl group of lactate at δ1.33

Key	Metabolites	Moieties	δ^1H (ppm) and multiplicity	CGA VS control
1	Lipoproteins	CH_3, CH_2CH_2C=	0.88(m), 1.28(m)	↑
2	L-Leucine	αCH, δCH_3, δCH_3	0.91(d), 0.96(d), 3.72(t)	–
3	L-Valine	αCH_3, βCH, γCH_3	0.99(d), 1.04(d)	–
4	L-Isoleucine	γCH_3, δCH_3	0.94(t), 1.01(d)	–
5	β-Hydroxybutyrate	γCH_3	1.22(d)	↓
6	L-Threonine	αCH, βCH, γCH_3	1.32(d), 4.25(m), 3.58(d)	–
7	Lactate	αCH, βCH_3	1.33(d), 4.11(q)	↓
8	L-Alanine	αCH, βCH_3	3.77(q), 1.48(d)	↓
9	Lipids	CH_2*CH_2CO, CH_2–C=C	1.58(m), 2.04(m)	–
		CH_2–C=O, CH–O–CO	2.24(m), 2.75(m)	–
10	L-Lysine	αCH, βCH_2, γCH_2, δCH_2	3.77(t), 1.89(m), 1.73(m)	↓
11	Acetate	CH_2–C=O	1.92(s)	–
12	Glycoprotein	CH_3–C=O	2.05(s), 2.08(m), 2.15(s)	–
13	Acetoacetate	CH_3, CH_2	2.29(s), 3.49(s)	↓
14	L-Glutamate	αCH, βCH_2, γCH2	3.75(m), 2.08(m), 2.37(m)	↓
15	Pyruvate	CH_3	2.37(s)	↓
16	Succinate	α, βCH_2	2.41(s)	↓
17	L-Glutamine	αCH, βCH_2, γCH_2	3.68(t), 2.15(m), 2.45(m)	↑
18	Citrate	CH_2	2.52(d), 2.70(d)	↓
19	Dimethylamine	CH_3	2.71(s)	↑
20	Trimethylamine	CH_3	2.92(s)	↑
21	Albumin	Lysyl-CH_2	3.02(s)	↓
22	Creatine	N–CH_3, CH_2	3.04(s), 3.93(s)	↓
23	Choline	N–$(CH_3)_3$, αCH_2, βCH_2	3.20(s), 4.05(t), 3.51(t)	↑
24	GPC	N–$(CH_3)_3$, OCH_2, NCH_2	3.22(s), 4.32(t), 3.51(t)	↑
25	TMAO	CH_3	3.26(s)	↑
26	Betaine	CH_3, CH_2	3.28(s), 3.90(s)	–
27	Taurine	N–CH_2, S–CH_2	3.27(t), 3.43(t)	–
28	Glycine	CH_2	3.56(s)	↑
29	L-Ornithine	CH_2, αCH	3.80(s), 3.79(t)	↑
30	β-Glucose	2-CH, 1-CH	3.25(dd), 4.65(d)	–
31	α-Glucose	1-CH	5.24(d)	–
32	Unsaturated lipids	=C–CH_2–C=, –CH=CH–	5.19(m), 5.31(m)	↑
33	L-Tyrosine	αCH, CH_2	7.20(d), 6.91(d)	↑
34	1-Methylhistidine	4-CH, 2-CH	7.05(s), 7.77(s)	↑
35	Formate	CH	8.45(s)	–

↓ and ↑, the metabolite levels are lower or higher, respectively, compared with the control group; –, the metabolite levels are the same as the control group

CGA VS control the CGA group compared to control group, *s* singlet, *d* doublet, *t* triplet, *q* quartet, *m* multiplet, *dd* doublet of doublets, *TMAO* trimethylamine-N-oxide, *GPC* glycerophosphoryl choline

alleviates hepatic ischemia and reperfusion-induced liver injury and that this protection is associated with an inhibition of inflammatory response and enhancement of anti-oxidant defense systems (Yun et al. 2012). Thus, in recent years, CGA has received much attention from biomedical scientists due to its beneficial effects as an anti-oxidant and hepatoprotective phytochemical. The hypocholesterolemic functions of CGA are probably due to an increase in fatty acid unitization in the liver (Wan et al. 2013), which utilizes long-chain fatty acids as major energy substrates (Dai et al. 2013a; Jobgen et al. 2006). However, the underlying mechanisms responsible for the hepatoprotective effect of CGA in animals remain unknown. Therefore, as an initial step to understand such mechanisms, the present study was conducted to identify biochemical metabolites as biomarkers for protective effects of CGA in rats.

Our metabolomic results indicate effects of CGA on energy and nitrogen metabolism in adult rats. Specifically, dietary supplementation with CGA decreased the concentrations of lactate, pyruvate, succinate, citrate, β-hydroxybutyrate and acetoacetate in the serum, as compared with

Table 5 Changes in relative concentrations of endogenous metabolites in aqueous soluble extracts from liver in CGA-supplemented rats on the basis of chemical shifts relative to TMSP at δ0.00

Key	Metabolites	Moieties	δ1H (ppm) and multiplicity	CGA VS control
1	Leucine	αCH, δCH_3, δCH_3	3.72(t), 0.91(d), 0.96(d)	–
2	Isoleucine	γCH_3, δCH_3	1.01(d), 0.94(t)	↑
3	Valine	αCH_3, βCH, γCH_3	3.61(d), 2.26(m), 0.99(d), 1.04(d)	–
4	β-Hydroxybutyrate	γCH_3	1.22(d)	–
5	Lactate	αCH, βCH_3	1.33(d), 4.11(q)	↑
6	Threonine	αCH, βCH, γCH_3	1.32(d), 4.25(m), 3.58(d)	–
7	Alanine	αCH, βCH_3	1.48(d), 3.77(q)	–
8	Acetate	CH_2–C=O	1.92(s)	–
9	Glutamate	αCH, βCH_2, γCH_2	2.08(m), 2.37(m), 3.75(m)	–
10	Glutamine	αCH, βCH_2, γCH_2	3.68(t), 2.15(m), 2.45(m)	–
11	Succinate	α, βCH_2	2.41(s)	↑
12	Glutathione	αCH, αCH_2, βCH_2, γCH_2	2.55(m), 2.16(m)	↑
13	Methylamine	CH_3	2.61(s)	–
14	Dimethylamine	CH_3	2.72(s)	–
15	Creatine	N–CH_3, CH_2	3.02(s), 3.93(s)	–
16	Creatinine	CH_3, CH_2	3.05(s), 4.05(s)	–
17	Choline	N–$(CH_3)_3$, αCH_2, βCH_2	3.2(s), 4.05(t), 3.51(t)	–
18	Phosphocholine	N–$(CH_3)_3$, βCH_2, αCH_2	3.21(s)	–
19	GPC	N–$(CH_3)_3$, OCH_2, NCH	3.23(s)	↓
20	TMAO	CH_3	3.27(s)	↓
21	Betaine	CH_3, CH_2	3.90(s), 3.28(s)	↓
22	Glycine	CH_2	3.56(s)	–
23	Taurine	N–CH_2, S–CH_2	3.41(t), 3.26(t)	–
24	β- Glucose	2-CH, 1-CH	3.25(dd), 4.65(d)	–
25	α-Glucose	1-CH	5.22(d)	–
26	Glycogen	CH	5.38–5.45(m), 3.35–3.4(m)	–
27	Uracil	N–CH	7.54(d), 5.81(d)	↓
28	Uridine	CH_2	7.87(d), 5.89(d), 3.92(dd)	↓
29	COP-choline	N–$(CH_3)_3$, αCH_2, βCH_2	7.95(s), 5.98(dd), 6.11(dd)	–
30	Adenosine	CH_2	8.22(s), 8.33(s), 6.06(d)	↓
31	Fumarate	CH, CH_3	6.53(s)	–
32	Tyrosine	αCH, βCH_2	7.18(d), 6.88(d)	–
33	Histidine	αCH, βCH_2	7.83(s), 7.08(s)	–
34	Hypoxanthine	N–CH, CH	8.19(s), 8.22(s)	–
35	Formate	CH	8.46(s)	↓
36	Nicotinate	CH_2	7.53(dd), 8.26(dt), 8.62(dd), 8.95(d)	↑

↓ and ↑, the metabolite levels are lower or higher, respectively, compared with the control group; –, the metabolite levels are the same as the control

CGA VS control the CGA group compared to control group, *s* singlet, *d* doublet, *t* triplet, *q* quartet, *m* multiplet, *dd* doublet of doublets, *TMAO* trimethylamine-N-oxide, *GPC* glycerophosphoryl choline

the control group (Table 4). Pyruvate is a key metabolite in glycolysis, amino acid metabolism, and the tricarboxylic acid (TCA) cycle (Kauppinen and Nicholls 1986). Lactate, which is formed from pyruvate and NADH by lactate dehydrogenase in the cytoplasm, is closely associated with energy metabolism and cellular redox state (Liu et al. 2011). The decreased concentrations of pyruvate and lactate in plasma may be related to an increased rate of hepatic gluconeogenesis and a reduced rate of anaerobic glycolysis in skeletal muscle (Krebs 1970). In addition, dietary supplementation with CGA affects the circulating levels of intermediates in the TCA cycle possibly due to the decreased concentrations of succinate and citrate in serum (Krebs and Lowenstein 1960; Krebs 1970). This suggests that the integrity of cells (e.g., hepatocytes and muscle) is improved by CGA supplementation, thereby reducing the release of intermediates of the TCA cycle into the extracellular space. Both acetoacetate and β-hydroxybutyrate are products of fatty acid oxidation in the liver (Wojtczak 1968; Wu et al. 1991). Interestingly, CGA supplementation decreased concentrations of acetoacetate and β-hydroxybutyrate in serum. This suggests a reduced rate of hepatic

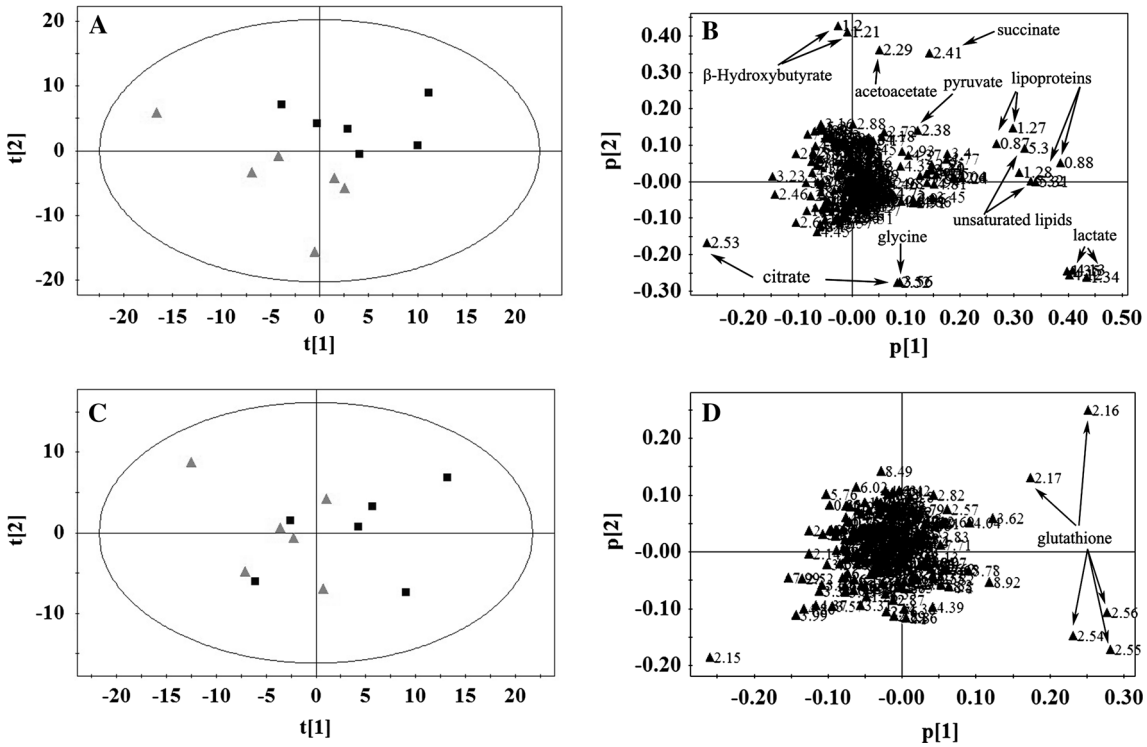

Fig. 3 PCA **a** scores plot and **b** loadings plot from ^{1}H NMR spectra of serum obtained from rats in the control group (*filled triangle*) and the CGA group (*filled square*). PCA **c** scores plot and **d** loadings plot from ^{1}H NMR spectra of aqueous soluble liver extracts obtained from rats in the control group (*filled triangle*) and the CGA group (*filled square*)

Table 6 Relative changes in concentrations of serum metabolites in CGA-supplemented rats as compared to the control group

Metabolites	NMR chemical shift (δ)	Change in direction	*P* values
Lipoproteins	0.88, 1.28	↑	0.042
β-Hydroxybutyrate	1.22	↓	0.049
Lactate	1.33, 4.11	↓	0.030
Acetoacetate	2.29	↓	0.037
Pyruvate	2.37	↓	0.015
Succinate	2.41	↓	0.027
Citrate	2.52	↓	0.011
Glycine	3.56	↑	0.017
Unsaturated lipids	5.31	↑	0.029

↓ Indicates a relative decrease, and ↑ indicates a relative increase in the integral value for the region containing the identified metabolite
Significant differences are set at $P < 0.05$

Table 7 Relative changes in concentrations of metabolites in aqueous soluble extracts from liver samples in CGA-supplemented rats as compared to the control group

Metabolites	NMR chemical shift (δ)	Change in direction	*P* value
Glutathione (reduced form)	2.55, 2.16	↑	0.023

↓ Indicates a relative decrease, and ↑ indicates a relative increase in the integral value for the region containing the identified metabolite
Significant differences are set at $P < 0.05$

ketogenesis and an increased rate of oxidation of ketone bodies by extrahepatic tissues, including skeletal muscle, heart and kidneys (Mubarak et al. 2013). CGA may have a beneficial effect on increasing mitochondrial biogenesis and oxidation, as reported for L-arginine (Satterfield and Wu 2011; Wu et al. 2012). An overall improvement in energy metabolism brought about by CGA supplementation may lead to increased cholesterol transport from peripheral tissues into the liver and the hepatic formation of the lipids beneficial for cardiovascular function and removal from the body. In support of this view, CGA supplementation increased the serum concentrations of unsaturated lipids and lipoproteins, as compared to the control group. Taken together, these results indicate that dietary supplementation with CGA can regulate energy expenditure in rats.

A striking finding from this study is the increased concentration of glutathione in the liver of CGA-supplemented rats, suggesting that CGA can promote the anti-oxidant activity. Glutathione is the major intracellular nonprotein thiol protecting cells against oxidative damage and harmful xenobiotics (Hayes and McLellan 1999; Schulz et al.

2000). Glutathione is synthesized in tissues and cells (e.g., liver and intestine) from glutamate, cysteine and glycine, and hepatocytes are the major producer and exporter of glutathione (Wu et al. 2004, Wu 2010). This finding is consistent with other studies in which CGA exerts a potent anti-oxidant effect in several animal models hepatic injury induced by CCl_4, lipopolysaccharide, or acetaminophen (Ji et al. 2013; Shi et al. 2009, 2013; Xu et al. 2010). In the present study, CGA supplementation increased hepatic concentrations of glutathione, indicating that CGA can promote glutathione synthesis in the liver. Glutathione also protects the small intestine (Wu et al. 2013a; Hou et al. 2012, 2013) and the large intestine (Wang et al. 2013a) from oxidative damage. We suggest that glutathione (reduced form) in serum may be a useful biomarker for the biological properties of CGA in vivo.

Another novel and unexpected finding from this work is that the concentration of glycine in serum was increased in CGA-supplemented rats (Table 6). This result is in agreement with the increased concentration of hepatic glutathione in the CGA group. In response to malnutrition and chronic diseases (e.g., starvation, diabetes, and obesity), concentrations of glycine in plasma are markedly reduced (Satterfield et al. 2012, 2013; Wu 2013b). Thus, CGA may be beneficial for treating or preventing these metabolic disorders. Available evidence shows that glycine contributes to improved anti-oxidative activity in humans and animals (Wang et al. 2013b). Thus, increasing dietary levels of glycine can enhance glutathione synthesis in tissues and protects tissues (e.g., the vascular bed and the intestine) against oxidative stress (Ruiz-Ramírez et al. 2014; Wang et al. 2014). Furthermore, oral glycine supplement attenuates oxidative damage in patients with metabolic syndrome (Díaz-Flores et al. 2013). Finally, glycine exhibits many biological properties, including metabolic regulation, anti-oxidative, cytoprotective, anti-inflammatory, and immunomodulatory properties (Cruz et al. 2008; El Hafidi et al. 2004; Mauriz et al. 2001).

Glycine is synthesized from threonine, serine, and hydroxyproline via inter-organ metabolism of amino acids (Wu 2013a; Wu et al. 2011). At present, the relative contributions of these pathways to endogenous glycine provision in animals remain unknown and should be quantified using isotopic techniques. An important role for intestinal microbes in providing glycine for absorption into the portal circulation should also be taken into consideration (Dai et al. 2011). This view is based on the recent findings that bacteria in the small intestine play an important role in the metabolism of amino acids, including glycine (Dai et al. 2010, 2012a, b, 2013b). It is possible that the increased level of glycine in the blood circulation may be explained by an ability of CGA to promote the endogenous synthesis of glycine in mammalian cells and intestinal microbes, as well as by enhanced absorption of glycine from the lumen of the gut. Traditionally, little attention has been paid to dietary requirement of glycine by animals (Wu et al. 2013b, 2014). Compared with many amino acids that are abundant in animal proteins, the content of glycine is relatively low in plant-based diets (Li et al. 2011; Dai et al. 2014). Under normal feeding conditions, the amount of glycine synthesized in vivo is insufficient to meet metabolic demands in birds and young mammals, particularly in a diseased state (Rezaei et al. 2013a, b; Wang et al. 2014; Wu et al. 2014). Although mild insufficiency of glycine is not threatening to survival of animals, a chronic shortage may result in impaired immune responses and other adverse effects on nutrient metabolism and health (Li et al. 2007; Matilla et al. 2002). Therefore, an increased concentration of glycine in serum or plasma can be another useful biomarker for the biological properties of CGA in vivo.

In summary, our results support the hypothesis that supplementation of CGA to a normal diet will regulate energy metabolism and enhance anti-oxidant function of rats. Dietary supplementation with CGA increased the concentrations of glycine in the serum and the concentrations of glutathione in the liver, thus promoting the anti-oxidant activity in the whole body. CGA also beneficially modulates energy metabolism in rats. We suggest that glycine and glutathione in the blood circulation are useful biomarkers for the biological effects of CGA in vivo. Further studies may be warranted to elucidate the underlying mechanisms and possible links between CGA and amino acid metabolism in animals.

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Conflict of interest The authors declare that they have no conflict of interests.

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