Acta Pharmacologica Sinica (2010) 31: 930–937
 1671-4083/10 $32.00
www.nature.com/aps

Original Article

Differential regulations of blood pressure and perturbed metabolism by total ginsenosides and conventional antihypertensive agents in spontaneously hypertensive rats

Ji-ye AA[1, #], Guang-ji WANG[1, *], Hai-ping HAO[1, #], Qing HUANG[2, #], Yi-hong LU[2, #], Bei YAN[3], Wei-bin ZHA[1], Lin-sheng LIU[1], An KANG[1]

[1]Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 21009, China; [2]Jiangsu Institute for Food and Drug Control, Nanjing 21008, China; [3]Department of Pharmacology, Beijing Hospital of Ministry of Health, Beijing 100730, China

Aim: To investigate the regulatory effects of total ginsenosides and the conventional antihypertensive agents (captopril, amlodipine, terazosin and hydrochlorothiazide) on the blood pressure and perturbed metabolism in spontaneously hypertensive rats (SHRs) and to analyze the cause-effect relationships between high blood pressure and the metabolic disorders of hypertension.
Methods: SHRs were administrated with total ginsenosides or the antihypertensive agents for eight weeks. Systolic blood pressure (SP) was measured every week and low-molecular-weight compounds in blood plasma were quantitatively analyzed using a nontargeted high-throughput metabolomic tool: gas chromatography/time of flight mass spectrometry (GC/TOFMS) . The metabolic patterns were evaluated using principal components analysis and potential markers of hypertension were identified.
Results: Total ginsenosides and the antihypertensive agents differentially regulated SP and the metabolic pattern in SHRs. Total ginsenosides caused a progressive and prolonged reduction of SP and markedly normalized the perturbed metabolism with 14 of 27 (51.8%) markers of hypertension which were regulated toward normal. Total ginsenosides also reduced free fatty acids' level toward normal levels. In contrast, captopril, amlodipine and terazosin efficiently depressed SP, but had little effect on metabolic perturbation with only 8 (29.6%), 4 (14.8%), and 4 (14.8%) markers, respectively, which were regulated.
Conclusion: The metabolic changes persisted when the blood pressure was lowered by the conventional antihypertensive agents, suggesting that hypertension may not be the cause of the metabolic perturbation in SHRs.

Keywords: metabonomics; hypertension; total ginsenosides; metabolic disorders; spontaneously hypertensive rats

Acta Pharmacologica Sinica (2010) 31: 930–937; doi: 10.1038/aps.2010.86

Introduction

Essential hypertension is the most prevalent chronic disease worldwide and is a major risk factor for cardiovascular morbidity and mortality[1–3]. Although previous studies have shown that essential hypertension is a multifactorial disease associated with metabolic disorders, its etiology and pathogenesis remain poorly understood[4, 5]. The "omics" technique offers a high-throughput screening strategy to explore the root causes of hypertension. The genome-wide mapping of human loci has linked many candidate genes to essential hypertension[6, 7], although none of them has been widely confirmed in different population groups. Recently, the metabolic profiling of hypertension has drawn the attention of researchers[8–14]. Brindle and colleagues found a relationship between serum metabolic profiles and blood pressure in clinic patients[9]. Our previous studies of clinical patients and spontaneously hypertensive rats (SHRs) clearly showed that metabolomic patterns associated with hypertension were different from those of the normotensive controls, demonstrating the obviously perturbed metabolism of hypertension[12–14]. Hypertension is inherently involved in metabolic syndrome, including hyperlipidemia and diabetes. The relationship between hypertension and metabolic disorders is of great interest. Unfortunately, although these studies have confirmed the metabolic perturbation underlying hypertension and underscore the potential of

# These authors contributed equally to this work.
* To whom correspondence should be addressed.
E-mail guangjiwang_cpu@hotmail.com
Received 2010-2-22 Accepted 2010-06-09

metabonomics to investigate the metabolic causes of hypertension, they have only investigated the hypertension and age-related metabolic patterns of SHRs and the influence of total ginsenosides on those patterns[9, 12, 13]. There has been little exploration of metabolism *in vivo* or the regulatory effects of ginseng on metabolism. Detailed metabolic variations and the regulatory effects of antihypertensive agents on metabolic disorders have not been investigated. The study of the metabolic effects of different types of antihypertensive agents is expected to yield comprehensive data that will provide key insights into the metabolic perturbation of hypertension and extend our understanding of the relationship between hypertension and metabolic syndrome.

Metabonomics represents a systematic approach to the study of metabolic phenotypes *in vivo*, thereby enhancing our understanding of systems biology[15]. Metabonomics can be defined as the measurement of the metabolome or the full set of low-molecular-weight endogenous compounds (or metabolites) present within cells, tissues, organisms, or biofluids that can reflect genetic modifications, exposure to pathogens, toxic agents, pharmaceuticals, and nutritional and environmental changes[16]. Rather than assessing a few compounds in a targeted analysis, metabonomics is a nontargeted method that analyzes as many low-molecular-weight compounds as possible, provided the sensitivity is sufficiently high. This set of metabolites is inherently involved in *in vivo* metabolic pathways or cycles. It is not only the starting point of the metabolic system, but also the endpoint of the metabolism that is involved in the physiological and metabolic responses to drug interventions. In recent decades, metabonomics has shown great utility in biomedical sciences and in the exploration of the mechanisms that underlie disease[8, 17–23].

In China, it has long been recognized that total ginsenosides (TG) can adjust blood pressure by balancing hyper- and hypotensive states[24–26]. Although dozens of investigations have reported the regulatory effects of ginseng extract or ginseng products on lipids and sugars, these studies have offered little information on the specific metabolites involved in these effects (with the exception of glucose and cholesterol). The reduction of systolic blood pressure (SP) and the regulation of metabolism *in vivo* mediated by TG and frontline clinical agents have never been thoroughly investigated and correlated. To study their regulatory effects on both blood pressure and the perturbed metabolism associated with hypertension, we used SHRs as the animal model in this study and explored their metabolic responses to TG using a metabonomic platform based on a gas chromatography/time-of-flight mass spectroscopy (GC/TOFMS) technique coupled to chemometric analysis[27–29]. Four commercially available antihypertensive agents, which act via diverse mechanisms, were included as positive antihypertensive control drugs.

Materials and methods

Chemicals

Authentic reference compounds were purchased from Sigma Aldrich (St Louis, MO, USA) or Sigma-Aldrich (Steinhein, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), or Serva (Heidelberg, Germany); all were of analytical or chromatography grade. Total ginsenosides were composed of the following: ginsenoside Rb1 (19.1%, *w/w*), Rb2 (13.8%), Rc (12.3%), Rd (9.7%), Re (11.8%), Rf (3.5%), and Rg1 (7.1%) (Jilin University, China). Amlodipine maleate (amlodipine), terazosin hydrochloride (terazosin), captopril, and hydrochlorothiazide were obtained from accredited pharmaceutical companies in China. The following were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), Fluka (Buchs, Switzerland), and Pierce Chemical Company, (IL, USA), respectively: stable-isotope-labeled internal standard (IS), [$^{13}C_2$]-myristic acid; alkane series (C_8–C_{40}), pyridine (silylation grade); and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA)+1% trimethylchlorosilane. Methanol, heptane, and sodium ethylenediaminetetraacetate (EDTA-Na) were of chromatography or analytical grades.

Animals and dosing

The animal studies were carried out after approval by the Animal Ethics Committee of China Pharmaceutical University. Male SHRs and normotensive control Wistar Kyoto rats (WKYs), all aged 8 weeks, were purchased from Wei Tong Li Hua Animal Center (Beijing, China). All rats were housed individually in metabolic cages in a standard animal laboratory with a 12-h light/dark cycle. Water and standard rat chow were available *ad libitum*. After 2 weeks of acclimatization, the rats were randomly divided into groups (n=6) based on body weight to receive treatment for 8 weeks (10–18 weeks of age). The following were administered to the rats based on the group to which they were assigned: vehicle (0.5% carboxymethycellulose sodium aqueous solution) or agents suspended in 1 mL of the vehicle, including total ginsenosides at 30 or 3 mg/kg (ip per day), captopril at 30 mg/kg (ig per day), amlodipine at 5 mg/kg (ig per day), terazosin at 5 mg/kg (ig per day), or hydrochlorothiazide at 10 mg/kg (ig per day). All of the doses were withdrawn after 8 weeks.

Measurement of SP and collection of plasma sample

The systolic blood pressures of conscious rats were measured using the indirect plethysmographic tail cuff (Shanghai Alcott Biotech Co, Ltd, China) every week as described previously[12]. After SP measurement, 0.5 mL of blood was collected in EDTA-Na anti-coagulated tubes, and the plasma was obtained by centrifuging at 1600x*g* for 10 min at 4 °C and stored at -70 °C until use.

Sample preparation and GC/TOFMS analysis

Plasma samples were thawed and the endogenous compounds were extracted, mathoximated, and trimethylsylilated as described previously[27]. To minimize systematic variations, the plasma samples were analyzed in random order. Using an Agilent 7683 autosampler, 1 μL of sample was injected into an Agilent 6890N system equipped with a fused silica capillary column chemically bonded with 0.18 μm DB5-MS stationary phase (10 m×0.18 mm ID). The inlet temperature was set to

250 °C, and helium was used as the carrier gas at a constant flow rate of 1.0 mL/min through the column. To achieve good separation, the column temperature was initially maintained at 70 °C for 2 min and then increased at a rate of 35 °C/min from 70 °C to 305 °C, where it was held for 2 min. The column effluent was introduced into the ion source of a Pegasus III MS (Leco Corp, St Joseph, MI, USA). The transferline temperature was set at 250 °C and the ion source temperature at 200 °C. Ions were generated by a 70-eV electron beam at a current of 3.2 mA. Masses were acquired with m/z 50–680 at a rate of 30 spectra/s.

Automatic peak detection and mass spectrum deconvolution were performed as described previously[27, 28]. To minimize interference from drug metabolites, only the peaks (of the same mass spectra and retention time/index) found in the blank control (*ie*, SHR control and WKY control without drug) were included in the data matrix for further data processing. The retention index of each peak was calculated by comparing the retention time of the peak with those of the alkane series C_8-C_{40}. The compounds were identified by comparison of the mass spectra and retention indices of all the detected compounds with the authentic reference standards and those available in the National Institute of Standards and Technology (NIST) library 2.0 (2005).

Principal component analysis and statistics

After normalization against the stable isotope internal standard, the data matrix was constructed with the observation/samples in columns and the responses/peaks as variables in rows. To improve the validity of the mathematical model, response variables of higher average standard deviation (>60%) within each group were excluded from the data matrix. Data processing was carried out using SIMCA-P 11 software (Umetrics, Umeå, Sweden). Principal component analysis (PCA) and partial least squares projection to latent structures & discriminant analysis (PLS-DA) were employed to process the acquired GC/TOFMS data, following established methodology[28–30]. PCA involves a mathematical procedure that transforms a number of correlated or uncorrelated variables into a smaller number of uncorrelated, new variables called "principal components" (PCs); in other words, it projects a K-dimensional space (K, the number of variables/peaks) and reduces it to a few PCs that describe the maximum variation in different groups or samples while retaining as much information as possible. Thus, the comparative analysis of data is greatly facilitated and can be visualized; for example, in two- or three-dimensional space. PCA is an unsupervised way to show the original scatter of the plots, whereas the supervised methods, partial least squares projection to latent structures (PLS) and discriminant analysis (DA), allow differentiation of the groups. The results of PCA or PLS-DA are displayed as score plots that represent the scatter of the samples. When tightly clustered, these indicate similar metabolic phenotypes. The relative positions of the samples suggest their similarity or dissimilarity; when loosely clustered, they indicate compositionally different metabolic phenotypes. The purpose of PLS-DA was to develop models that differentiate groups or classes. Samples from the same groups were classified into a single group for PLS_DA modeling. Cross-validation with seven cross-validation groups was used throughout to determine the number of principal components[31].

The following statistics for the regression models are discussed throughout this paper. R^2X is the percentage of all GC/TOFMS response variables explained by the model, R^2Y is the percentage of all observation/sample variables explained by the model, and Q^2Y is the percentage of all observation/sample variables predicted by the model. The range of these parameters is between 0 and 1, with values approaching 1 indicating better explanation or prediction. Statistical analysis between groups was validated for biochemical parameters or metabolite concentrations using one-way ANOVA with a significance level of 0.05 or 0.01.

Results

SP of SHR treated with TG, amlodipine, captopril, terazosin, or hydrochlorothiazide

We clearly observed that the SP of the SHR control group increased gradually from week 10 to week 20, whereas the SP of the normotensive control group remained stable (Figure 1). As the model SHRs age ranged from 10 to 20 weeks, their SP remained steady at a high level, 30%–50% higher than that of the normotensive WKY. The SP of the animals was measured every week after treatment with TG, amlodipine, captopril, terazosin, or hydrochlorothiazide. SP decreased rapidly and significantly in the amlodipine-, captopril-, and terazosin-treated groups, whereas TG and hydrochlorothiazide reduced SP only slightly (Figure 1). Interestingly, SP was elevated during the first week of treatment in the TG group. As the treatment continued, SP continued to decrease slowly in all groups, although the captopril-treated group showed a sharp decline in SP after six weeks of treatment. Eight weeks later, we stopped all treatments and SP increased quickly in all groups, with the exception of the TG group. TG displayed its greatest antihypertensive effect on SP in week 8, when SP was 15.6% lower than that of the SHR controls. Two weeks after the cessation of treatment, the average increase in SP was only 8.0% of the TG group, which was still 8.5% lower than the SP of the SHR controls (P<0.01, one-way ANOVA). Although the SP of the captopril group reached the same level as that of the TG group, the percentage increase was much greater, 29.6% higher than TG at week 8. Clearly, TG yielded a persistent and sustained long-term reduction in SP, even two weeks after the cessation of treatment. This result suggests that TG acts on an unknown target to lower SP.

Metabolic phenotype of SHR and the regulatory effects of TG

According to the PCA algorithm, every dot represents a sample and contains information regarding all the metabolites measured in the sample as well as their concentrations. The metabolites and their concentrations determine the sample's position in the scores plot. Therefore, the relative positions of the plots suggest similarities or differences among the sam-

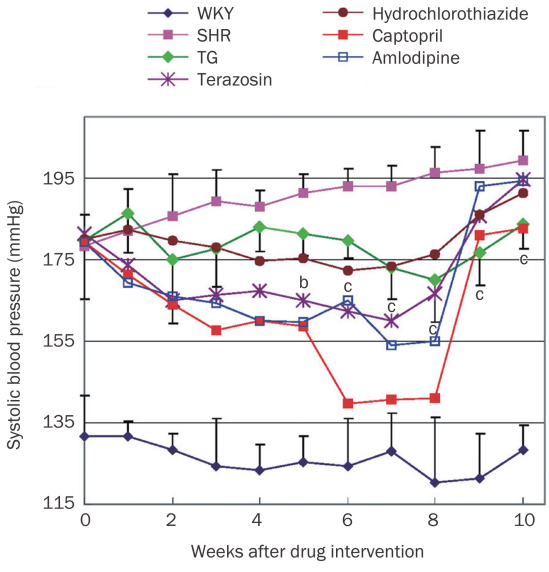

Figure 1. Systolic blood pressure (SP) measured from zero to eight weeks of treatment with total ginsenosides (TG), terazosin, captopril, amlodipine, or hydrochlorothiazide, and for two weeks after withdrawal of the drugs. Treatment with TG, amlodipine, captopril, terazosin, and hydrochlorothiazide led to a rapid reduction in SP. This effect was significant in the amlodipine-, captopril-, and terazosin-treated groups, whereas TG and hydrochlorothiazide exhibited a trend towards reduced SP. Two weeks after the withdrawal of the drugs, the SP of the animals in all groups increased rapidly, with the exception of those in the TG-treated groups. $^{b}P<0.05$, $^{c}P<0.01$ significantly different from SHR (one-way ANOVA).

ples; the closer the dots, the more similar the metabolite compositions and concentrations within the samples. Conversely, the further apart the dots, the greater the differences among the samples. To evaluate the effects of the therapeutic agents on the metabolic phenotype, a PLS-DA model was calculated between the SHRs and the WKY normotensive controls between weeks 10 and 18 (*ie*, zero, two, four, six, and eight weeks after treatment with the agents). This produced a good PLS-DA model of four principal components (PC1: $R^2X=0.302$, $R^2Y=0.140$, $Q^2Y=0.123$; PC2: $R^2X=0.510$, $R^2Y=0.304$, $Q^2Y=0.214$; PC3: $R^2X=0.536$, $R^2Y=0.387$, $Q^2Y=0.291$; and PC4: $R^2X=0.576$, $R^2Y=0.423$, $Q^2Y=0.298$; Figure 2A), revealing a clear separation of SHRs and WKYs and a continual movement in the age-related variation from week 10 to week 18.

From week 10 to week 18, the plots moved gradually from the bottom to the top of the figures, for both SHRs and WKYs, indicating continual metabolic modifications with aging. However, the development of hypertension resulted in the repositioning of the SHR plots further from the WKY plots, suggesting that the metabonomic variations became increasingly significant.

Although the SHR data points were clearly separated from those of the normotensive controls and moved further away with time, the profiles of the SHRs treated with TG showed a gradual movement away from the starting point at the lower right of the positive control and SHR plots toward the upper left, closer to that of the normotensive WKY controls (Figure 2A). The close clustering of the TG group and the WKY group eight weeks after the treatment strongly suggests a similarity between the two groups in terms of their metabolic phenotypes (metabolite compositions and concentrations). This movement pathway suggests a gradual adjustment of the endogenous compounds in the SHRs toward those in the normotensive control. Because endogenous metabolites are intrinsically involved in biochemical processes *in vivo*, this finding suggests that TG regulates the metabolic disorders that correlate with hypertension.

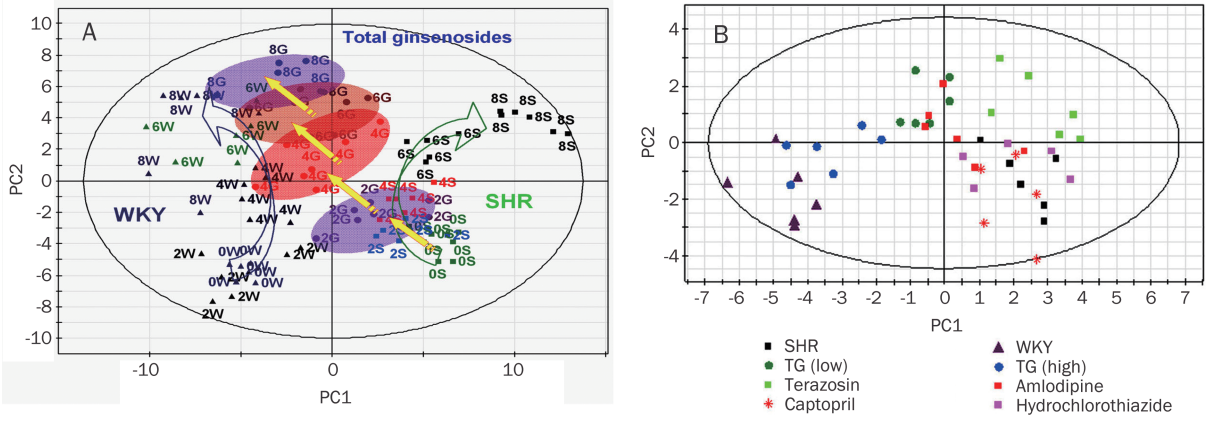

Figure 2. A) The scores plot shows the metabolomic profiles of SHR, WKY, and SHR treated with total ginsenosides (TG) for two, four, six, and eight weeks. Treatment with TG led to a gradual repositioning of the plots from SHR and toward WKY, suggesting a clear rectification of the metabolome. B) The scores plot shows the metabolomic patterns for the total ginsenosides (TG)-, terazosin-, captopril-, amlodipine-, and hydrochlorothiazide-treated groups at week 8. Significantly different metabolic phenotypes were observed after eight weeks of treatment. The TG group clustered close to WKY, whereas the other groups overlapped with or were close to SHR, which suggests the rectification of endogenous metabolites after TG treatment and a lesser regulatory effect after treatment with the other drugs.

Comparative regulatory effects of TG, amlodipine, captopril, terazosin, and hydrochlorothiazide on the metabolic phenotype

The regulatory effects on the metabolism of the antihypertensive agents were also compared. Treatment with TG, amlodipine, captopril, terazosin, or hydrochlorothiazide for four weeks resulted in a shift from the SHR control toward the WKY phenotype in the TG-treated group and a slight shift in the amlodipine-treated group. However, the effects observed in the other groups were minor (data not shown). The continuation of the treatments for eight weeks resulted in more pronounced trends.

A snapshot of the clustering of the treated groups revealed a clear differentiation between the treated SHRs and the SHR and WKY controls (Figure 2B). The PLS-DA model of the three principal components (R^2X, 0.621; R^2Y, 0.439; Q^2Y, 0.286) shows that the TG group clustered close to the normotensive control; this result suggests a metabonomic similarity. The amlodipine-treated group was located far from both the SHR and the WKY controls, suggesting a partial regulatory effect on the metabonomic profile. In contrast, the groups treated with captopril, terazosin, or hydrochlorothiazide clustered close to the SHRs control, but far from the WKY control, suggesting only a minor regulation of metabolism. To investigate the dose-dependent metabolic response, we also examined the effects of a low dose of TG. Our data demonstrate that in terms of the observed metabolic adjustment, a low dose of TG was less effective than a high dose of TG.

Regulation of endogenous molecules and the metabolic network

To investigate the metabolic pathways and the specific compounds regulated by the TG intervention, we identified compounds that exhibited differential variation in SHRs and WKYs. In total, we identified 27 compounds (potential markers) that differed significantly between SHRs and WKYs (Table 1). These compounds included free fatty acids (FFAs), amino acids, lipids, and low-molecular-weight organic acids. The variations in their levels suggested the abnormal metabolism of lipids and the tricarboxylic acid cycle (TCA) and abnormali-

Table 1. The relative abundance of the identified compounds differentiating SHR from WKY and the regulation by TG. [b]$P<0.05$, [c]$P<0.01$ statistically different from that in SHR (one-way ANOVA); [e]$P<0.05$, [f]$P<0.01$ statistically different from that in WKY (one-way ANOVA).

Identified compounds	WKY (n=6)		SHR (n=6)		SHR/WKY		TG group (n=6)		Regulatory effects	
	Mean	SD	Mean	SD	FOLD change	P	Mean	SD		
Oleic acid	1783945	337017	4733098	481490	2.65	0.000	227102	603653	c	↓
Linoleic acid	3455190	1609054	5734207	1091390	1.66	0.017	4653098	1200381		↓
Palmitic acid	2364923	379592	4409830	367411	1.86	0.000	3112846	634977		↓
9-(Z)-Hexadecenoic acid	281275	97867	551580	78654	1.96	0.000	309967	114154	c	↓
Arachidonic acid	475498	41903	593878	72439	1.25	0.013	459250	40556		↓
Campesterol[#]	150357	91935	340035	71032	2.26	0.003	382256	114206	f	/
beta-Sitosterol[#]	328143	143306	525743	103495	1.60	0.021	598850	117818	e	/
Cholesterol	7377553	1739904	4911862	1304910	0.67	0.020	5177279	1230574	e	/
Glycerol-2,3-Diphosphate	1333058	324454	691553	165510	0.52	0.002	1116494	161409	c	↑
Glycerol-3-phosphate	1843532	663979	1089775	151531	0.59	0.038	2254995	274214	c	↑
alpha-Ketoglutarate	639815	105195	943346	177174	1.47	0.023	558065	52848	c	↓
Fumarate	56558	17288	23209	11045	0.41	0.007	62617	11880	c	↑
Citrate	6794945	1670787	10006047	2414382	1.47	0.023	7131960	2774474		↓
Pyruvate	2156930	659838	3294852	574616	1.53	0.043	1815504	1290360	b	↓
3-Hydroxybutyrate	1314319	263739	3523070	1247738	2.68	0.015	865614	395038	c	↓
Aconitate	32415	26817	91165	34527	2.81	0.008	34801	13678	c	↓
^{1}H-Indole-3-propanoate	706281	128660	66858	19455	0.09	0.000	263513	43231	b, e	↑
Glycerate	378533	126459	204937	44122	0.54	0.018	328657	42359	e	↑
Serine	4813791	360330	3220286	387138	0.67	0.000	3386430	878641	e	/
Threonine	6178436	273524	3838460	402535	0.62	0.000	4046089	1267995	e	/
Ornithine	1302239	246639	885944	236574	0.68	0.014	1207453	554479		↑
Tryptophan	3982887	439055	8532228	2019938	2.14	0.002	4974343	1407934	b	↓
Cystine	270690	55960	583558	138531	2.16	0.000	300416	89341	b	↓
Glutamate	1303700	395647	2504625	655325	1.92	0.021	1526047	433673	b	↓
Cysteine	318078	58155	426023	69316	1.34	0.047	259833	36831	c	↓
Creatinine	186448	81306	343095	100844	1.84	0.036	208144	51567		↓
Glucose	60432950	8604010	91531617	19466227	1.51	0.005	69789343	15931253		↓

All peaks areas were normalized by the stable isotope internal standard, [$^{13}C_2$]-myristic acid;

↑, ↓, /: the compounds level was up-, down-, or not regulated by TG intervention.

[#]: Although not endogenous compounds, their abundances differentiate between SHR and WKY.

ties in glucose and amino acid turnover.

Treatment with TG led to the restoration of 14 (51.8%) of the 27 discriminatory metabolites (Table 1) to normal levels, that is, significantly different from those of the SHR control ($P<0.05$, one-way ANOVA) but not different from those of the normotensive WKY control ($P>0.05$, one-way ANOVA). Amlodipine, captopril, terazosin, and hydrochlorothiazide adjusted the levels of eight (29.6%), four (14.8%), four (14.8%), and two (7.4%) metabolites toward normal values, respectively. In contrast, an analysis of the regulatory effects of the specific agents revealed that captopril significantly regulated α-ketoglutarate, tryptophan, cystine, and cysteine to the normal levels observed in WKY. Amlodipine rectified all the metabolites regulated by captopril, and citrate, pyruvate, and creatinine. Terazosin adjusted tryptophan, cystine, cysteine, and creatinine, whereas hydrochlorothiazide adjusted 3-hydroxybutyrate and cystine.

The regulatory effects of these antihypertensive agents were also reflected in the diverse regulation of specific compounds. For example, high-dose TG adjusted the level of 9-(Z)-hexadecenoic acid to normal (*ie*, to a similar level observed in WKYs), which was significantly different from its level in SHRs. In contrast, the other agents did not significantly regulate 9-(Z)-hexadecenoic acid (Supplementary Information, Figure S1A). Interestingly, TG regulated most FFAs to normal or near normal levels. High-dose TG, amlodipine, and captopril reduced the levels of α-ketoglutarate to normal, whereas the other drugs had a smaller effect (Figure S1B). Only TG and hydrochlorothiamide markedly reduced the levels of 3-hydroxybutyrate, whereas the other drugs had minor effects (Figure S1C). The regulation of arachidonic acid, fumarate, and ornithine by TG was also observed (Figure S1D–F). In general, TG, terazosin, amlodipine, captopril, and hydrochlorothiazide had different effects on the regulation of 9-(Z)-hexadecenoic acid, arachidonic acid, α-ketoglutarate, 3-hydroxybutyrate, fumarate, and ornithine.

Correlation between SP and potential hypertension markers

The results discussed above show that many of the potential hypertension markers are regulated by TG. It is important to determine whether there is a quantitative relationship between SP and these markers. Linear regression between the SP values and the normalized peak areas of the metabolites revealed that many lipids (*eg*, oleic acid, palmitic acid, and 9-(Z)-hexadecenoic acid) correlated with SP, whereas several amino acids (*eg*, serine, threonine, and ornithine) correlated inversely with SP (Supplementary file, Figure S2). This suggests that these compounds are not only potential markers of hypertension, but also quantitative markers of the therapeutic response to these antihypertensive agents.

Discussion

Although metabolic dysfunction is closely related to hypertension, the cause of hypertension remains unknown and the cause-effect relationships between hypertension and metabolic disorders have not been established. In this study, we not only provided evidence of the marked perturbation of lipid and glucose metabolism in SHR but also found that TG normalizes this metabolic perturbation and shows mild, sustained antihypertensive effects; in contrast, the conventional antihypertensive agents efficiently reduced SP but had only minor regulatory effects on the perturbed metabolism. Captopril, amlodipine, and terazosin act on different targets to reduce the peripheral resistance of blood vessels and hence reduce blood pressure. This is illustrated by the following example: captopril acts on the angiotensin-converting enzyme; amlodipine selectively inhibits calcium ion influx across cell membranes, thus acting directly on vascular smooth muscles; terazosin produces a hypotensive effect mainly by blocking the α_1 adrenoceptors; and hydrochlorothiazide is a diuretic agent that interferes with the renal tubular mechanism to reduce electrolyte reabsorption. Unlike TG, these agents significantly reduced SP, but had little regulatory effect on the perturbed metabolism of SHR, even after the hypertension had been reduced for eight weeks. The significant regulation of the perturbed metabolism of SHRs resulted primarily from the normalization of glucose metabolism and lipid metabolism, whereas the effects of the other agents on the perturbed metabolism of SHRs were probably associated with their efficient reduction of SP. The withdrawal of the drugs led to a rapid rebound of SP. These results confirm that these agents act on hypertension at a superficial level. Because the efficient reduction of hypertension had only a slight effect on the perturbed metabolism, hypertension may not be the cause of this perturbation of the metabolism.

SHRs are genetically defective in fatty acid translocase/CD36[32]. Studies have confirmed that defective CD36 contributes to insulin resistance and increased serum levels of fatty acids in SHR and humans[33, 34]. The identified metabolites that differentiate SHRs from WKYs (Table 1) suggest perturbations in the metabolism of lipids, glucose, and the TCA cycle as well as amino acid turnover (Figure S3). The elevation of FFAs in SHRs confirms the genetic defect in the fatty acid translocase/CD36 gene. Theoretically, the downregulated expression of the fatty acid translocase/CD36 gene will reduce the β-oxidation of FFAs and therefore the animal's energy supply. To our surprise, we detected a marked elevation of 3-hydroxybutyrate, one of the ketone body oxidation products of FFA, possibly because SHRs tend to utilize energy sources other than ketobodies. As supporting evidence, we observed the elevation of TCA cycle intermediates in SHRs relative to those in WKYs. This finding indicates that SHRs rely more on the TCA cycle for energy than on lipid metabolism. High levels of glucose always favor the synthesis of lipids[35], and we observed higher levels of FFA and glucose in SHR. However, SHRs are smaller and thinner than WKYs. This obvious contradiction suggests that both the catabolism and the anabolism of lipids are retarded in SHRs.

In general, the TG treatment not only led to a prolonged reduction in SP, even two weeks after its withdrawal, but also showed a distinct regulation of the metabolic pattern (Figure

2A, 2B) and restored the levels of most FFAs in SHRs toward normal (Table 1). These results suggest that TG acts on fatty acid translocase and indicates a relationship between the SP-lowering effects of TG and its regulation of the perturbed metabolism of SHRs. Brindle and colleagues were able to distinguish the serum samples from patients with low/normal SP from those with borderline or high SP. However, the borderline- and high-SP samples were indistinguishable from each other[9]. This result indicates that the metabolic phenotypes of borderline and high-SP patients are similar and that metabolic disorders have developed in borderline patients. In other words, metabolic disorders develop before high SP can be determined, and the occurrence of metabolic disorders does not immediately result in high SP. These lines of evidence may explain why TG treatment clearly regulated the metabolism of SHR but did not immediately lower SP. These results also provide indirect support for the hypothesis that TG exerts its antihypertensive effect by normalizing the perturbed metabolism involved in hypertension and that metabolic perturbation may play an important role in the development of hypertension. If this hypothesis is correct, the regulation of the perturbed metabolism will be a novel target for the treatment and cure of hypertension.

An infusion of FFAs results in elevated blood pressure in both animals and humans[36–43]. A large-scale population study concluded that the elevation of FFAs in human individuals is a highly significant risk factor for the subsequent development of hypertension[44]. In the present study, we not only determined the presence of higher levels of FFAs in SHRs but also found a good correlation between SP and FFAs (*eg*, oleic acid, 9-(Z)-hexadecenoic acid, and palmitic acid). Together with evidence from other animal studies and large-scale studies in human subjects, these results suggest that the levels of FFA or FFA-related metabolism may play an important role in the development and remission of hypertension and that FFA may be a good biomarker or indicator of hypertension. Based on the fact that TG significantly downregulated most FFA toward normal levels and produced a mild and sustained reduction in SP, even two weeks after its withdrawal, we propose that TG exerts its antihypertensive effects via the regulation of FFA-mediated biochemical processes.

In summary, the five agents that were investigated produced different hypotensive effects and distinct regulation patterns of the perturbed metabolism that occurs in SHRs. Captopril, amlodipine, and terazosin efficiently reduced SP but had only a minor regulatory effect on the perturbed metabolism of SHRs, indicating that the efficient reduction of hypertension did not result in the correction of metabolic perturbation and that metabolic perturbation is not the consequence of hypertension. Total ginsenosides regulated the perturbed metabolism and showed prolonged hypotensive effects, even two weeks after its withdrawal. These results suggest that metabolic perturbation plays an important role in the development of hypertension and that TG is a unique antihypertensive agent that can regulate perturbed metabolism.

Acknowledgements

This study was financially supported by the National Key New Drug Creation Special Programs (2009ZX09304-001 and 2009ZX09502-004), the National Natural Science Foundation of China (30630076), the Jiangsu Province Social Development Foundation (BE2008673), the Jiangsu Nature Science Fund (BK2008038), and the National 11th 5-Year Technology Supporting Program of China (2006BAI08B04).

Author contribution

Ji-ye AA, Guang-ji WANG, and Hai-ping HAO designed the research; Qing HUANG, Yi-hong LU, Bei YAN, Wei-bin ZHA, Lin-sheng LIU, and An KANG performed the research; Ji-ye AA, Qing HUANG, Yi-hong LU, Bei YAN, and Wei-bin ZHA analyzed the data; Ji-ye AA and Qing HUANG wrote the paper.

References

1 Yach D, Leeder SR, Bell J, Kistnasamy B. Global chronic diseases. Science 2005; 307: 317.
2 Kearney PM, Whelton M, Reynolds K, Whelton PK, He J. Worldwide prevalence of hypertension: a systematic review. J Hypertens 2004; 22: 11–9.
3 Jones DW, Hall JE. World Hypertension Day 2007. Hypertension 2007; 49: 939–40.
4 Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. Cell 2001; 104: 545–56.
5 Charchar FJ, Zimmerli LU, Tomaszewski M. The pressure of finding human hypertension genes: new tools, old dilemmas. J Hum Hypertens 2008; 22: 821–8.
6 Cowley AW Jr. The genetic dissection of essential hypertension. Nat Rev Genet 2006; 7: 829–40.
7 Caulfield M, Munroe P, Pembroke J, Samani N, Dominiczak A, Brown M, *et al*. Genome-wide mapping of human loci for essential hypertension. Lancet 2003; 361: 2118–23.
8 Holmes E, Loo RL, Stamler J, Bictash M, Yap IK, Chan Q, *et al*. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature 2008; 453: 396–401.
9 Brindle JT, Nicholson JK, Schofield PM, Grainger DJ, Holmes E. Application of chemometrics to ^{1}H NMR spectroscopic data to investigate a relationship between human serum metabolic profiles and hypertension. Analyst 2003; 128: 32–6.
10 Akira K, Imachi M, Hashimoto T. Investigations into biochemical changes of genetic hypertensive rats using ^{1}H nuclear magnetic resonance-based metabonomics. Hypertens Res 2005; 28: 425–30.
11 Akira K, Masu S, Imachi M, Mitome H, Hashimoto M, Hashimoto T. ^{1}H NMR-based metabonomic analysis of urine from young spontaneously hypertensive rats. J Pharm Biomed Anal 2008; 46: 550–6.
12 Lu YH, A J, Wang G, Hao H, Huang Q, Yan B, *et al*. Gas chromatography/time-of-flight mass spectrometry based metabonomic approach to differentiating hypertension- and age-related metabolic variation in spontaneously hypertensive rats. Rapid Commun Mass Spectrom 2008; 22: 2882–8.
13 Lu YH, Wang GJ, Huang Q, A JY, Hao HP, Hao K. Investigations into metabonomic profiling of spontaneously hypertensive rat and metabolic effects of total ginsenoside on SHR using GC/MS. Chin J Nat Med 2007; 5: 443–7.
14 Lu Y, Hao H, Wang G, Chen H, Zhu X, Xiang B, *et al*. Metabolomics approach to the biochemical differentiation of traditional Chinese

medicine syndrome types of hypertension. Chin J Clin Pharmacol Ther 2007; 12: 1144–50.
15 Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol 2004; 22: 245–52.
16 Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999; 29: 1181–9.
17 Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, *et al*. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ^{1}H-NMR-based metabonomics. Nat Med 2002; 8: 1439–44.
18 Sabatine MS, Liu E, Morrow DA, Heller E, McCarroll R, Wiegand R, *et al*. Metabonomic identification of novel biomarkers of myocardial ischemia. Circulation 2005; 112: 3868–75.
19 Denkert C, Budczies J, Kind T, Weichert W, Tablack P, Sehouli J, *et al*. Mass spectrometry-based metabolic profiling reveals different metabolite patterns in invasive ovarian carcinomas and ovarian borderline tumors. Cancer Res 2006; 66: 10795–804.
20 Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, *et al*. Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci USA 2008; 105: 2117–22.
21 Nicholson JK, Lindon JC. Systems biology: metabonomics. Nature 2008; 455: 1054–6.
22 Holmes E, Wilson ID, Nicholson JK. Metabolic phenotyping in health and disease. Cell 2008; 134: 714–7.
23 Clayton TA, Lindon JC, Cloarec O, Antti H, Charuel C, Hanton G, *et al*. Pharmaco-metabonomic phenotyping and personalized drug treatment. Nature 2006; 440: 1073–7.
24 Yue PY, Mak NK, Cheng YK, Leung KW, Ng TB, Fan DT, *et al*. Pharmacogenomics and the Yin/Yang actions of ginseng: anti-tumor, angiomodulating and steroid-like activities of ginsenosides. Chin Med 2007; 2: 6
25 Zhang Y, Li KQ. Two-side adjustment mechanism of traditional Chinese medicine. Acta J Henan Univ Chin Med 2003; 18: 15–7.
26 Nie K. Two-side adjustment effect of Chinese medicines. New J Tradit Chin Med 2000; 32: 3–5.
27 A J, Trygg J, Gullberg J, Johansson AI, Jonsson P, Antti H, *et al*. Extraction and GC/MS analysis of the human blood plasma metabolome. Anal Chem 2005; 77: 8086–94.
28 Jonsson P, Johansson AI, Gullberg J, Trygg J, AJ, Grung B, *et al*. High-throughput data analysis for detecting and identifying differences between samples in GC/MS-based metabolomic analyses. Anal Chem 2005; 77: 5635–42.
29 Trygg J, Holmes E, Lundstedt T. Chemometrics in metabonomics. J Proteome Res 2007; 6: 469–79.
30 Eriksson L, Johansson E, Kettaneh-Wold N, Wold S. Multi- and megavariate data analysis principles and applications. Umeatrics Academy, Umetrics AB, Sweden. 2001.
31 Wold S. Cross-validatory estimation of the number of components in factor and principal components models. Technometrics 1978; 20: 397–405.
32 Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, *et al*. Identification of Cd36 (fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. Nat Genet 1999; 21: 76–83.
33 Pravenec M, Landa V, Zidek V, Musilova A, Kren V, Kazdova L, *et al*. Transgenic rescue of defective Cd36 ameliorates insulin resistance in spontaneously hypertensive rats. Nat Genet 2001; 27: 156–8.
34 Miyaoka K, Kuwasako T, Hirano K, Nozaki S, Yamashita S, Matsuzawa Y. CD36 deficiency associated with insulin resistance. Lancet 2001; 357: 686–7.
35 Thomas M. Devlin. Textbook of biochemistry with clinical correlations, 6th edition. Wiley-Liss, Hoboken, New Jersey, 2006.
36 Bulow J, Madsen J, Hojgaard L. Reversibility of the effects on local circulation of high lipid concentrations in blood. Scand J Clin Lab Invest 1995; 50: 291–6.
37 Grekin RJ, Dumont CJ, Vollmer AP, Watts SW, Webb RC. Mechanisms in the pressor effects of hepatic portalvenous fatty acid infusion. Am J Physiol 1997; 273: R324–30.
38 Grekin RJ, Vollmer AP, Sider RS. Pressor effects of portal venous oleate infusion. A proposed mechanism for obesity hypertension. Hypertension 1995; 26: 193–8.
39 Steinberg HO, Paradisi G, Hook G, Crowder K, Cronin J, Baron AD. Free fatty acid elevation impairs insulin-mediated vasodilation and nitric oxide production. Diabetes 2000; 49: 1231–8.
40 Steinberg HO, Tarshoby M, Monestel R, Hook G, Cronin J, Johnson A, *et al*. Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. J Clin Invest 1997; 100: 1230–9.
41 Stojiljkovic MP, Zhang D, Lopes HF, Lee CG, Goodfriend TL, Egan BM. Hemodynamic effects of lipids in humans. Am J Physiol Regul Integr Comp Physiol 2001; 280: R1674–9.
42 Lopes HF, Martin KL, Nashar K, Morrow JD, Goodfriend TL, Egan BM. DASH diet lowers blood pressure and lipid-induced oxidative stress in obesity. Hypertensio 2003; 41: 422–30.
43 Lopes HF, Stojiljkovic MP, Zhang D, Goodfriend TL, Egan BM. The pressor response to acute hyperlipidemia is enhanced in lean normotensive offspring of hypertensive parents. Am J Hypertens 2001; 14: 1032–7.
44 Fagot-Campagna A, Balkau B, Simon D, Warnet JM, Claude JR, Ducimetière P, *et al*. High free fatty acid concentration: an independent risk factor for hypertension in the Paris Prospective Study. Int J Epidemiol 1998; 27: 808–13.