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Metabolic Fate of Tea Polyphenols in Humans

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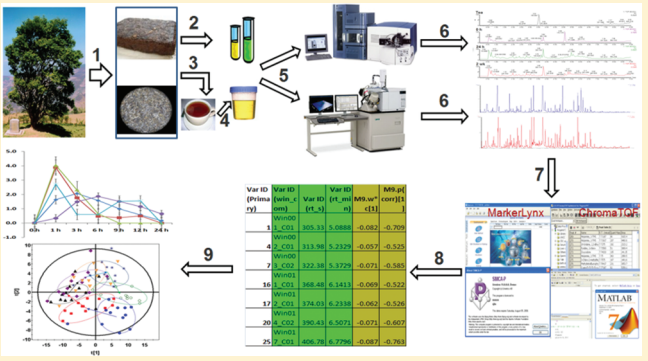
Supporting Information

ABSTRACT: Polyphenols, a ubiquitous group of secondary plant metabolites sharing at least one aromatic ring structure with one or more hydroxyl groups, represent a large group of natural antioxidants abundant in fruits, vegetables, and beverages, such as grape juice, wine, and tea, and are widely considered to contribute to health benefits in humans. However, little is yet known concerning their bioactive forms *in vivo* and the mechanisms by which they may alter our metabolome, which ultimately contribute toward disease prevention. Here we report a study to determine the metabolic fate of polyphenolic components in a Chinese tea (Pu-erh) in human subjects using a metabonomic profiling approach coupled with multivariate and univariate statistical analysis. Urine samples were collected at 0 h, 1 h, 3 h, 6 h, 9 h, 12 h, and 24 h within the first 24 h and once a day during a 6 week period including a 2 week baseline phase, a 2 week daily Pu-erh tea ingestion phase, and a 2 week "wash-out" phase, and they were analyzed by gas chromatography mass spectrometry and liquid chromatography mass spectrometry. The dynamic concentration profile of bioavailable plant molecules (due to *in vivo* absorption and the hepatic and gut bacterial metabolism) and the human metabolic response profile were measured and correlated with each other. This study demonstrates that the metabonomic strategy will enable us to integrate the overwhelming amount of metabolic end points as a systems' response to the absorption, metabolism, and disposition of a multicomponent botanical intervention system, leading to a direct elucidation of their mechanisms of action.



KEYWORDS: *metabolic fate, polyphenols, metabonomics, metabolomics, multicomponent nutraceuticals, urine, tea, Pu-erh tea*

INTRODUCTION

The past two decades have witnessed an increasing application of botanical-based nutraceuticals as complementary interventions against a number of conditions such as metabolic syndromes and cancer.[1,2] The vast number of phytochemical entities including primary and secondary plant metabolites present in natural products, also known as the plant metabolome, and their huge concentration range are inextricable obstacles for nutraceutical/drug evaluation and development. The pharmacology of botanical-based nutraceuticals entails a "network" approach, in which multiple compounds interact with multiple targets *in vivo* with interdependent activities to achieve an optimal effect.[3,4] The traditional approach to understanding the pharmacology of a multicomponent nutraceutical is to study the effects of a single component on a single biological reaction, enzyme, gene, etc., and gradually assemble those effects into an integrated picture. However, assembling the results obtained from such a reductionistic approach to achieve a systems understanding of a concerted pharmacological intervention has proven impractical.[5] Additionally, the pharmacokinetic properties of a given compound in a multicomponent assay may be significantly different from that in a single compound assay, due to drug−drug interactions. With such a complex "network" approach involving a large number of multiparametric variables, it is technically challenging to identify the origin of each significantly changed metabolite in the global metabolite pool

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and to assess the human (endogenous) biochemical responses to the exposure of these exogenous (xenobiotic) compounds.

We all possess a unique metabolic profile characterized by a panel of endogenous metabolites and acquired exogenous metabolites resulting from our daily consumption of food (such as fruits and vegetables) and supplements. This "metabolic footprint" conditions an individual drug response and personalized intervention strategy. Recently we proposed an integrated metabonomic profiling strategy for the pharmacokinetics and pharmacodynamics studies of multicomponent drugs using tandem mass spectrometry (MS).[6] A plant based nutraceutical intervention should be regarded as a process in which a plant metabolome interacts with our biological system, encompassing the genome, proteome, and metabolome. When plant derived compounds, such as a group of tea polyphenols, enter into our body, significant changes will occur in the metabolite compositions in our blood pool/urine in a time-dependent manner. The resulting metabolome will then be comprised of (1) a group of exogenous compounds absorbed in our circulating system, (2) a group of exogenous compounds transformed by our hepatic enzymes and gut microbes, and (3) a group of endogenous metabolites that are significantly altered in response to the intake of the plant derived compounds. In recent years a few studies have addressed the metabolic fate of polyphenols in animals and humans and have stressed the importance of the microbial metabolism of polyphenols.[7−10] The central hypothesis of our study is that the metabolic fate of dietary tea components involves dynamic absorption and biotransformation in the gut-liver metabolic axis, and interactions between the plant metabolome and mammalian biological systems will result in a time-dependent alteration in the mammalian metabolic pathways in response to the absorption and biodegradation of plant components *in vivo*.

Pu-erh tea, a fermented tea containing a large array of polyphenolic constituents,[11] has a wide range of biological and pharmaceutical properties.[12,13] Studies to better understand the bioefficacy and the formation and biological activity of polyphenol metabolites, identify the biological signatures, and study the polyphenols with a systems biology approach are being supported by the National Center for Complementary and Alternative Medicine (NCCAM).[14] Metabonomics[15] or metabolomics,[16] with its capability of simultaneously analyzing hundreds and thousands of variables, is uniquely suitable for simultaneously measuring multiple nutraceutical components (bioavailable plant metabolite profile) *in vivo*, as well as identifying the characteristic mammalian metabolic response profile in humans exposed to nutraceuticals.[17−20] Utilizing a metabonomics platform to interpret the efficacies or toxicities of herbal medicines has been a key focus of recent herbal and pharmaceutical research.[21−25] Here, we report an integrated profiling approach coupled with multivariate and univariate statistical analysis that evaluates the metabolic fate of a dietary intervention with Pu-erh tea in 20 human subjects. Urine samples were collected at different time points and analyzed by gas chromatography mass spectrometry and liquid chromatography mass spectrometry to characterize the dynamic tea metabolite profile and mammalian metabolic response profile in a global metabolite pool resulting from the dietary intervention.

MATERIALS AND METHODS

Chemicals and Materials

The chemicals and Pu-erh tea used in this study were identical to those used in our previous study[13] (see the Supporting Information).

Ethics Statement

This study was conducted in accordance with the ethical guidelines and approved by the research ethics committee of the School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China. Written informed consent was obtained from all the subjects before study initiation (Consent Form is provided in the Supporting Information).

Participants and Study Protocol

Approximately 1 kg of 5-year-old Pu-erh tea was mixed to produce a homogeneous sample, and then it was ground into a fine powder and filtered through a 20 mesh screen. The Pu-erh tea was prepared by infusing 10 g of powder in 200 mL of boiling water for 10 min, followed by straining. Participants received a dose equivalent to 5 cups of commercially prepared tea. Twenty healthy men (n = 10) and women (n = 10), whose mean age was 25 ± 2 years (range 22−32), were enrolled in this study.

The volunteers did not consume tea and polyphenol-rich diets prior to the experiment and fasted overnight before Pu-erh tea intervention. They were provided with the standard meals three times per day (Supporting Information Table S1) during the experiment. Spot urine samples of all 20 participants were collected daily between 11:00 and 11:30 a.m. during a 6 week period that included a 2 week baseline phase, a 2 week daily Pu-erh tea ingestion phase, and a 2 week postdosing phase. Urine samples of 12 (randomly selected from the 20 participants) healthy men (n = 6) and women (n = 6) were collected just before breakfast including the 200 mL of Pu-erh tea and at 1 h, 3 h, 6 h, 9 h, 12 h, and 24 h thereafter. Participants reported no adverse effects after the ingestion of Pu-erh tea. Urine samples were stored at −80 °C before analysis.

Tea and Urine Sample Analysis by UPLC-QTOFMS Analysis

The tea infusion used in this experiment was centrifuged, and a volume of 500 μL of ultrapure water was added to the tea (500 μL) and vortexed for 1 min, and then the supernatant was filtered through a syringe filter (0.22 μm) for UPLC-QTOFMS analysis according to our previous report.[13]

Urine samples were processed according to our previously published work.[13] The collected urine samples were centrifuged at 13,000 rpm for 10 min at 4 °C, and the resulting supernatants were immediately stored at −80 °C pending UPLC-QTOFMS analysis. Ultrapure water (500 μL) was added to urine (500 μL) and vortexed for 1 min, and then the sample was filtered through a syringe filter (0.22 μm) for UPLC-QTOFMS analysis.

Tea and urine metabolite profiling were performed using a Waters ACQUITY UPLC system equipped with a binary solvent delivery manager and a sample manager (Waters Corporation, Milford, MA, USA), coupled to a Micromass Q-TOF Premier mass spectrometer equipped with an electrospray interface (Waters Corporation, Milford, MA, USA), according to our previous published work (for details, see the Supporting Information).[21]

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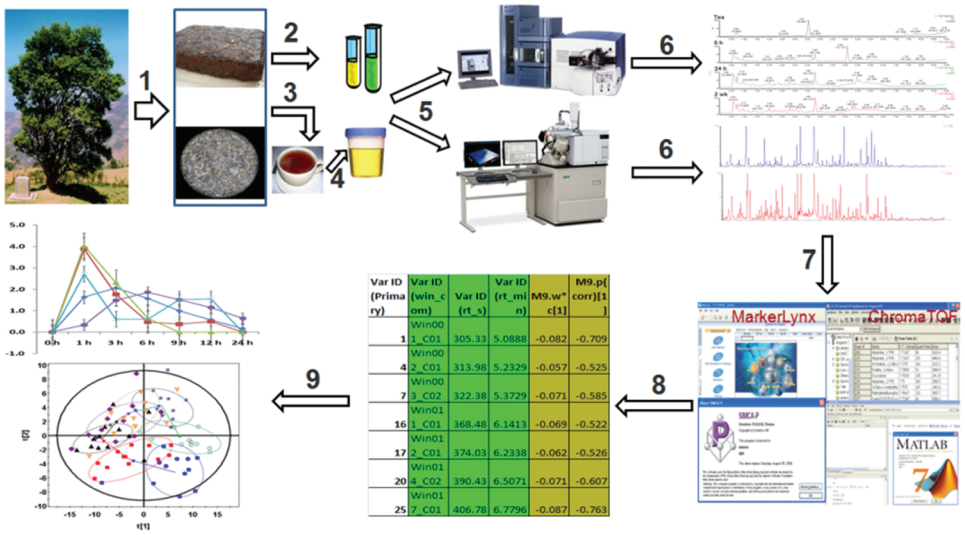

Figure 1. Flow chart of the integrated metabonomics and pharmacokinetics approach for the study of Pu-erh tea. (1) Prepare Pu-erh tea from tea leaves; (2) Extract tea for MS analysis; (3) Prepare tea infusion for human intervention; (4) Collect urine samples at different time points for MS analysis; (5) UPLC-QTOFMS and GC-TOFMS analysis for tea and urine samples; (6) Data acquisition; (7) Data analysis with multivariate and univariate statistical methods; (8) Peak list including intact tea polyphenols, metabolites of the absorbed polyphenols, and altered endogenous metabolites; (9) Data interpretation and visualization.

Tea and Urine Analysis by GC-TOFMS

Tea and urine analysis by GC-TOFMS were performed according to our previous published work with minor modification (for details, see the Supporting Information).[26,27] Briefly, each 1 μL aliquot of the derivatized solution was injected into an Agilent 6890N gas chromatograph in splitless mode coupled with a Pegasus HT time-of-flight mass spectrometry and separated on a DB-5 ms capillary column (30 m × 250 μm i.d., 0.25 μm film thickness; (5%-phenyl)-methylpolysiloxane bonded and cross-linked; Agilent J&W Scientific, Folsom, CA) with helium as the carrier gas at a constant flow rate of 1.0 mL/min (for details, see the Supporting Information).

UPLC-QTOFMS Data Analysis

The UPLC-QTOFMS data from the urine samples was analyzed to identify potential discriminant variables. The ES+ raw data was analyzed by the MarkerLynx applications manager version 4.1 (Waters, Manchester, U.K.) using parameters reported in our previous work.[21] The resulting three-dimensional data sets were imported into the SIMCA-P+ 12.0 Software package (Umetrics, Umeå, Sweden) for multivariate statistical analysis such as principle component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA). For details, see the Supporting Information.

GC-TOFMS Data Analysis

The acquired MS files from GC-TOFMS analysis were exported in NetCDF format by ChromaTOF software (v3.30, Leco Co., CA, USA). CDF files were extracted using custom scripts (revised Matlab toolbox HDA, developed by Jonsson et al.[28,29]) in the MATLAB 7.1 (The MathWorks, Inc., USA) for data pretreatment procedures such as baseline correction, denoising, smoothing, and alignment; time-window splitting; and peak feature extraction (based on multivariate curve resolution algorithm).[29] The resulting three dimension data set, including sample information, peak retention time, and peak intensities, was imported into the SIMCA-P+ 12.0 Software package (Umetrics, Umeå, Sweden) for data analysis according to our previous published work.[26,27] For details, see the Supporting Information.

Analysis of Pu-erh Tea-Induced Metabolic Variations

Characterization of the dynamic Pu-erh tea profile and mammalian metabolic response profile using an integrated MS-based global profiling approach was conducted with minor modifications to the process as proposed in our published paper.[6] The study protocol was described in the Participants and Study Protocol section. A controlled diet was provided to all participants throughout the study to minimize diet-induced metabolic "noise". The Pu-erh tea was assayed and characterized. Two analytical platforms, LC-MS and GC-MS, were used for urine sample analysis to measure the plant derived components as well as the significantly altered endogenous metabolites.

A "pre-dose metabonome" of the participants was obtained from the analysis of urine samples at time-point 0 prior to the tea intake. The entire differential compounds ("altered endogenous metabolites") in urine at a postdose time point were selected by comparing the compounds in postdose (time-point 1) urine samples with the predose urinary metabonome using a univariate statistical analysis, Student's t test. The plant metabolome was derived from the chemical profiling of Pu-erh tea. Similarity analysis techniques (achieved with Microsoft Office Access 2007; for details, see the Supporting Information) were used to identify the shared variables between plant metabolome and postdose variables. The shared variables are actually the compounds in the urine sample that were absorbed from Pu-erh tea, as characterized by accurate mass (m/z) and retention time (Rt) in the LC-MS spectra. The shared variables between the predose metabonome of individuals and the postdose variables are the endogenous metabolites altered as a result of tea exposure. After exclusion of the two sets of the shared variables (intact polyphenols, endogenous metabolites), the remaining of the postdose variables are the metabolites of the absorbed polyphenols derived from Pu-erh tea. The identified bioavailable plant compounds, the intact polyphenols and the metabolites of the absorbed polyphenols, can be further

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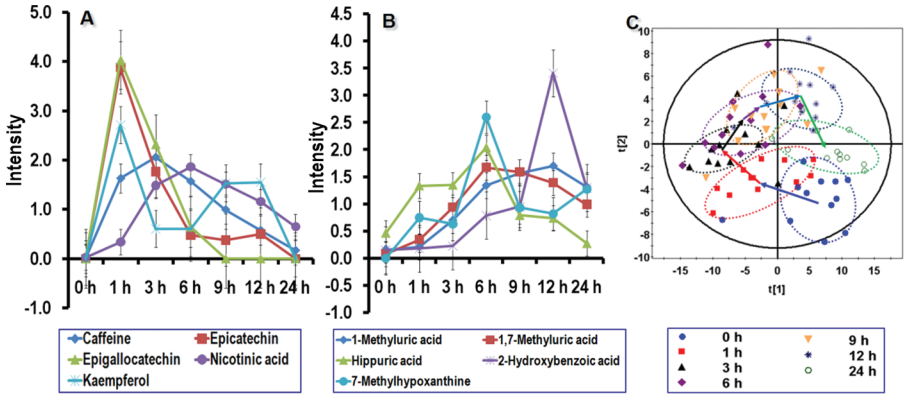

Figure 2. Urine concentration−time courses of some representative substances—intact polyphenols, metabolites of the absorbed polyphenols, and altered endogenous metabolites, after Pu-erh tea intake. (A) Urine concentration−time courses of intact Pu-erh tea polyphenols. (B) Urine concentration−time courses of the metabolites of the absorbed tea polyphenols. (C) A time-dependent trajectory of metabolite profiles at different time points after Pu-erh tea intake. The PCA scores plot showed a time dependent trajectory of urinary metabolites which clustered at different spatial positions and time points.

investigated at different time points for metabolic fate characterization of Pu-erh tea.

Correlation Analysis

Metabolites of interest with high linear relationship were found using the Pearson correlation ($|r| \geq 0.7$) when pairwise metabolite vectors were compared at a certain time point, such as intact polyphenols vs metabolites of the absorbed polyphenols derived from Pu-erh tea or metabolites of the absorbed polyphenols derived from Pu-erh tea vs differentially expressed endogenous metabolites from urine samples at 24 h. Similarly, to study the relationship of the dynamic response of metabolites along with the time course, the mean value of each metabolite was calculated at each time point (0 h → 1 h → 3 h → 6 h → 9 h → 12 h → 24 h). A new metabolite vector with 7 mean values calculated at 7 different time points, representing the average response, was constructed, and the Pearson correlation coefficients were calculated similarly with the pairwise comparison of metabolites.

RESULTS

UPLC-QTOFMS and GC-TOFMS Profiles of Pu-erh Tea and Urine Samples

The untargeted MS profiling of Pu-erh tea and urine samples was conducted following the scheme shown in Figure 1. Representative base peak intensity (BPI) chromatograms of UPLC-QTOFMS (A) and total ion current (TIC) chromatograms of GC-TOFMS (B) are shown in Supporting Information Figures S1 and S2. The PCA scores plot generated with the combined UPLC-QTOFMS and GC-TOFMS data showed a time dependent trajectory of urinary metabolites which clustered at different spatial positions and time points (Figure 2C and Figure S3).

A total of 5,636 and 392 features were detected from the UPLC-QTOFMS and GC-TOFMS spectral data sets, respectively, for each urine sample, and a total of 647 and 428 features from the water extract of the Pu-erh tea were obtained from the two analytical platforms, respectively. The Student's *t* test was performed on all urinary features derived from UPLC-QTOFMS and GC-TOFMS and calculated at different time points before and after Pu-erh tea exposure. The variables selected were those with statistical significance ($p < 0.05$) between predose and postdose samples at each time point of 1 h, 3 h, 6 h, 9 h, 12 h, and 24 h. A total of 2,476 significant variables from UPLC-QTOFMS and 176 from GC-TOFMS were selected, with a *p* value less than 0.05 at least once at all time points.

Compound Annotation

Among the 2,476 significantly altered features from UPLC-QTOFMS analysis, 796 were identified by searching against the HMDB library with accurate mass, and 132 were further verified by available reference standards. Analogously, among the 176 significantly altered compounds from GC-TOFMS data, 167 were identified with NIST 05 standard mass spectral databases (NIST, Gaithersburg, MD) with a similarity of greater than 70% and 58 were further verified by available standards.

As described in the Experimental Section of Analysis of Pu-erh Tea-Induced Metabolic Variations, a panel of 19 and 26 compounds were defined as intact polyphenols and metabolites of the absorbed polyphenols from Pu-erh tea using the similarity analysis technique by comparing the retention time and accurate mass of the variables obtained from UPLC-QTOFMS and retention time and five principal fragment ions of GC-TOFMS. A panel of 118 compounds are the altered human endogenous metabolites resulting from Pu-erh tea intake. Representative metabolites with the retention time, *p* value and fold change (FC) are provided in Table 1. Complete lists of the three data sets are shown in Tables S2−4.

Dynamic Concentration Profiles of Intact Polyphenols and Metabolites of the Absorbed Polyphenols

The urine concentration (intensity)−time courses of representative substances−intact polyphenols and metabolites of the absorbed polyphenols after Pu-erh tea intake are illustrated in Figure 2. The concentrations of the intact polyphenols, epigallocatechin, and caffeine reached maximum levels in urine at 1 h after oral administration (Figure 2A). These metabolites were cleared away from urine at 9 h postdose, as in previous studies.[20] Kaempferol presents two peaks in the urine profile at 1 and 9 h, respectively. This finding is consistent with previous PK results of this compound,[30] presumably due to enterogastric[31] and enterohepatic circulations.[32] Figure 2B shows the concentration profiles of several representative metabolites of the absorbed polyphenols. 1,7-Dimethyluric acid, hippuric acid, and 7-methylhypoxanthine reached maximum

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Table 1. Representative Intact Polyphenols, Metabolites of the Absorbed Polyphenols, and the Altered Endogenous Metabolites under Pu-Erh Tea Intervention[a]

			FC						*p* value					
no.	metabolite	retention time (min)	1 h−0 h	3 h−0 h	6 h−0 h	9 h−0 h	12 h−0 h	24 h−0 h	1 h−0 h	3 h−0 h	6 h−0 h	9 h−0 h	12 h−0 h	24 h−0 h
Intact Pu-erh Tea Polyphenols														
1	1,3-dimethyluric acid† (U)	2.17	4.25	11.56	20.54	19.48	17.09	12.14	4.65×10^{-1}	9.88×10^{-2}	1.71×10^{-2}	7.45×10^{-3}	4.37×10^{-2}	2.06×10^{-1}
2	caffeine† (U)	3.60	454.37	573.74	437.21	277.02	160.06	46.28	9.34×10^{-6}	5.29×10^{-9}	7.67×10^{-8}	3.30×10^{-9}	1.50×10^{-5}	5.84×10^{-4}
3	epigallocatechin* (U)	2.95							1.21×10^{-1}	3.53×10^{-2}	3.28×10^{-1}	1.00	1.00	1.00
4	nicotinic acid† (U)	2.96	14.31	62.95	78.98	63.95	49.02	27.31	2.11×10^{-5}	1.25×10^{-8}	1.14×10^{-7}	1.71×10^{-11}	2.51×10^{-11}	4.86×10^{-7}
5	theobromine† (U)	2.60	4.04	6.76	7.75	5.42	4.43	2.67	4.39×10^{-3}	9.48×10^{-6}	1.57×10^{-6}	1.56×10^{-6}	2.92×10^{-5}	1.70×10^{-2}
6	theophylline† (U)	2.96	11.62	42.69	54.55	45.99	38.67	19.22	2.75×10^{-6}	1.50×10^{-8}	9.11×10^{-8}	$1.98 \times 10^{-1}0$	1.94×10^{-11}	6.03×10^{-7}
7	4-aminobutanoic acid* (G)	16.56	3.95	5.14	24.12	39.08	100.29	49.03	6.42×10^{-2}	1.35×10^{-1}	4.17×10^{-3}	5.72×10^{-3}	1.66×10^{-4}	3.78×10^{-3}
8	3,5-hydroxybenzoic acid‡ (G)	21.70	2.03	3.48	1.54	0.42	0.47	0.20	1.77×10^{-2}	1.56×10^{-6}	1.01×10^{-1}	2.15×10^{-2}	3.74×10^{-2}	1.63×10^{-3}
Metabolites of the Absorbed Pu-erh Tea Polyphenols														
1	1-methyluric acid† (U)	2.01	1.35	4.43	8.50	9.98	10.78	8.33	1.48×10^{-2}	5.36×10^{-4}	2.47×10^{-6}	3.99×10^{-8}	7.21×10^{-8}	6.82×10^{-9}
2	1,7-methyluric acid† (U)	2.17	4.25	11.56	20.54	19.48	17.09	12.14	7.38×10^{-1}	9.84×10^{-2}	4.52×10^{-2}	1.03×10^{-1}	4.76×10^{-1}	8.91×10^{-1}
3	1-methylxanthine† (U)	1.49							1.0	1.61×10^{-1}	7.83×10^{-2}	9.88×10^{-2}	4.33×10^{-3}	1.99×10^{-4}
4	3-hydroxyphenylacetic acid† (U)	7.03	4.36	5.84	6.29	2.04	3.09	3.77	5.23×10^{-2}	3.09×10^{-2}	2.52×10^{-2}	5.22×10^{-1}	2.29×10^{-1}	2.15×10^{-1}
5	paraxanthine† (U)	1.49	0.00	4.27	11.43	24.53	27.51	27.14	3.28×10^{-1}	3.41×10^{-1}	6.20×10^{-2}	2.20×10^{-3}	2.40×10^{-4}	5.26×10^{-5}
6	hippuric acid* (U)	16.83	2.86	2.89	4.37	1.69	1.59	0.59	5.91×10^{-1}	1.80×10^{-1}	6.04×10^{-1}	3.78×10^{-1}	1.62×10^{-2}	4.17×10^{-1}
7	2-hydroxybenzoic acid‡ (G)	27.82	1.16	1.48	5.16	6.23	22.34	8.49	7.90×10^{-1}	5.56×10^{-1}	1.14×10^{-1}	2.47×10^{-2}	2.12×10^{-2}	2.45×10^{-2}
Altered Endogenous Metabolites														
1	valine* (U)	0.90	2.58	1.25	2.16	2.32	2.76	1.38	1.88×10^{-1}	7.35×10^{-1}	1.95×10^{-1}	1.34×10^{-1}	3.14×10^{-2}	5.76×10^{-1}
2	4-hydroxy-3-methoxyphenylacetic acid* (G)	21.63	0.39	0.11	0.27	0.55	0.85	1.12	6.76×10^{-4}	1.19×10^{-6}	2.21×10^{-5}	9.54×10^{-3}	4.01×10^{-1}	5.18×10^{-1}
3	ornithine‡ (G)	24.90	0.51	0.33	1.36	4.29	5.10	2.90	2.64×10^{-3}	3.40×10^{-4}	1.70×10^{-1}	2.32×10^{-4}	8.84×10^{-7}	1.43×10^{-4}
4	2-methoxyphenol‡ (G)	8.11	0.48	0.31	0.31	0.41	0.59	0.61	4.87×10^{-2}	7.01×10^{-3}	5.80×10^{-3}	1.56×10^{-2}	8.79×10^{-2}	1.04×10^{-1}
5	4-aminobutanoic acid* (G)	16.56	3.95	5.14	24.12	39.08	100.29	49.03	6.42×10^{-2}	1.35×10^{-1}	4.17×10^{-3}	5.72×10^{-3}	1.66×10^{-4}	3.78×10^{-3}
6	aminomalonic acid‡ (G)	12.26	1.90	3.09	3.09	0.45	0.57	0.35	2.00×10^{-1}	2.35×10^{-3}	8.04×10^{-3}	7.00×10^{-2}	1.62×10^{-1}	3.19×10^{-2}
7	phenol* (G)	5.63	1.04	1.22	1.43	1.14	1.32	2.22	8.44×10^{-1}	1.70×10^{-1}	1.07×10^{-1}	4.72×10^{-1}	2.29×10^{-1}	9.04×10^{-5}

[a]Metabolites are annotated using the following: *, available reference standards; †, accurate mass measurement with the aid of web-based resources, such as the Human Metabonome Database and METLIN (http://metlin.scripps.edu); ‡, the mass fragments with those present in commercially available mass spectral databases such as NIST, Wiley, and NBS, with a similarity threshold of 70%. U means data obtained from UPLC-QTOFMS analysis; G means data obtained from GC-TOFMS analysis.

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levels in urine 6 h postdose. A time-dependent trajectory of (endogenous) urinary metabolite profiles at different time points after Pu-erh tea intake is shown in Figure 2C. In the PCA map, each spot represents a sample, and each assembly of samples indicated a particular metabolic profile at different time points. The locus marked by arrows represents the spatial location of the center of a metabolite cluster changing with the time, starting from the predose assembly. From Figure 2C, urinary metabolite profiles at different time points showed a distinct difference from that at the "predose" time point. Furthermore, the metabolite profile at 24 h is approaching the predose profile, suggesting that the metabolic homeostasis was being restored.

Effect of Pu-erh Tea Intake on Human Metabolite End Points

Metabonomic response profiles at 24 h, 2 week, and 2 week wash-out to Pu-erh tea intervention are depicted as a heat map in Figure 3 by means of Matlab 7.1 software. The differentially

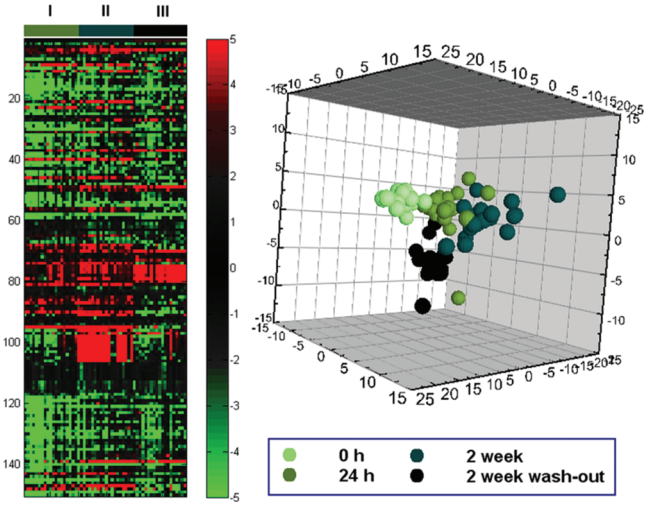

Figure 3. Effect of Pu-erh tea intake on human metabolite end points. (A) Heatmap showing differences in altered endogenous metabolites detected from the metabonome after Pu-erh tea intake (postdose) as compared to predose metabonome. (I) metabonomic changes at 24 h postdose relative to predose; (II) 2 week postdose vs predose; (III) 2 week wash-out vs predose. Each cell in the heat map represents the fold change between the two time points (e.g., postdose vs the predose) for a particular metabolite. It visualizes the level of each metabolite in each sample ranging from high (red) over average (black) to low (green). (B) 3-D PCA scores plot of urinary metabolic profiles at predose, 24 h postdose, 2 week postdose, and 2 week wash-out postdose.

expressed (endogenous) metabolites in response to Pu-erh tea intake were selected by multivariate statistical models, orthogonal partial least-squares-discriminant analysis (OPLS-DA), and S-plot, with the very important parameters in the projection (VIP) greater than 1.0 and absolute p(corr) value greater than 0.5 (Supporting Information Figure S4).[24,33] The representative metabolites most significantly altered in urine are listed in Supporting Information Table S5 along with fold changes. The urinary metabolite profile at 2 weeks of Pu-erh tea daily intake showed a distinct difference from that at the "predose" time point in Figure 3. The metabolic difference is primarily due to the altered gut microbial-human cometabolism, including the increased urinary excretion of 4-methoxyphenylacetic acid, inositol, and 5-hydroxytryptophan, and decreased concentration of 3-chlorotyrosine, 2-aminobenzoic acid, and 2,5-dihydroxy-1H-indole. The change of these metabolites and their metabolic pathways seems to be consistent with the cholesterol and plasma triglyceride reducing effects of Pu-erh tea.[34,35]

The metabolic profile of the subjects (black dots in Figure 3) after 2 week wash out showed a recovery trend but was distinct from that at the predose state, suggesting an incomplete recovery after the washout phase. Disruption of the gut microbial populations by Pu-erh tea could at least partially account for this result, as evidenced by altered metabolites such as 4-methoxyphenylacetic acid, 5-hydroxytryptophan, 3,4-dimethylbenzoic acid, 2-ethoxyphenol, and hydrocaffeic acid (see Supporting Information Table S5), associated with gut microbial cometabolism.[36,37]

Correlation of Intact Polyphenols, Metabolites of the Absorbed Polyphenols, and Endogenous Metabolite Markers

The correlation among the intact polyphenols, metabolites of the absorbed polyphenols, and the altered endogenous metabolites was established in Figure 4, with positive (red) and negative (blue) (here, $r \geq 0.7$ or $r \leq -0.7$) values. In general, intact polyphenols are positively corrected with their metabolites, whereas the urinary concentration of endogenous metabolites either increased or decreased in response to the alteration of the bioavailable tea metabolites. Caffeine, for example, was positively corrected with its biodegradated metabolites, paraxanthine, theophylline, hippuric acid, and 3-hydroxyphenylacetic acid. Paraxanthine was positively corrected with ornithine, valine, tyrosine, and 2-methylguanosine, whereas theophylline was positively corrected with 2-methylguanosine but negatively corrected with urea and aminomalonic acid. The increase in urine concentration of tea metabolite, 3-hydroxyphenylacetic acid, resulted in the elevated level of aminomalonic acid and 2-aminobutyric acid.

DISCUSSION

This study showed that a 2 week intervention with Pu-erh tea produces significant changes in the urinary global metabolites profiles. The combined UPLC-QTOFMS and GC-TOFMS-based metabonomics approach provided valuable and complementary information on polyphenol degradation in the human body and its dynamic impact on human metabolome. The key technical challenge for determining the metabolic fate of tea polyphenols is the differentiation among three panels of variables: the intact polyphenols, the metabolites of the absorbed polyphenols, and the altered human endogenous metabolites. The three panels of variables can be annotated with the available reference standards in our lab, web-based resources such as the Human Metabolome Database (http://www.hmdb.ca/),[38] and mass spectrometry based metabonomics databases[39] such as NIST. In-depth interrogation and characterization of these variables in m/z and retention time are required during the data analysis to avoid false-positive results. A strong quality control protocol used in sample preparation and analysis eliminating the compositional variation of Pu-erh tea and the dynamic analytical variations during the instrumental analyses will also minimize false positive or false negative results in the differential variables identified from the data analysis.

With multiple time points chosen in the study design for sample collections, a semiquantitative time course for each of

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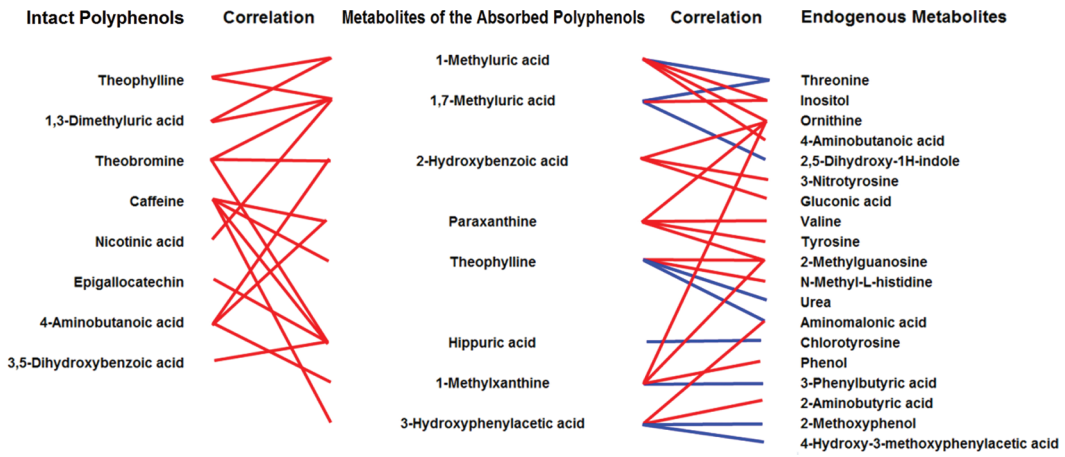

Figure 4. Correlation of intact polyphenols, metabolites of the absorbed polyphenols, and altered endogenous metabolites in response to Pu-erh tea exposure. The relationships among the three groups of compounds were visualized in the form of correlation maps, which are displayed by red (positive) or blue (negative) lines.

the identified bioavailable plant components (absorbed *in vivo*) and their derivatives (metabolized *in vivo*) was obtained to reflect a complete metabolic profile of the Pu-erh tea preparation. This has been partially demonstrated in a previous publication.[18] Preferably, a metabonomic measurement is needed to correlate the fluctuation of bioavailable plant metabolome with the response of human metabonome, which demands much greater bioanalytical strength and bioinformatics effort. The time-dependent changes of some representative substances (Table 1), intact polyphenols and metabolites of the absorbed polyphenols, are shown in Figure 2. The relationships among intact polyphenols, metabolites of the absorbed polyphenols, and altered endogenous metabolites in response to Pu-erh tea exposure were established using the Pearson correlation analysis, as shown in Figure 4. These results substantially advanced our understanding of the metabolic fate of a multicomponent nutraceutical as well as the dynamic response of our human metabolic system.[40]

The metabonomic changes can be regarded as a drug response profile consisting of "pharmacodynamic" end points, which can be used to evaluate the pharmacological or beneficial effects of a specific dietary or botanical drug intervention. As shown in Figures 2 and 3, the dynamic changes and the relationships among intact tea polyphenols, metabolites of the absorbed polyphenols, and altered endogenous metabolites are supported by previously published literature. An example is that the increased nicotinic acid levels in the urine after Pu-erh tea intake may be responsible for the cholesterol reducing and the lipid-lowering effects of Pu-erh tea, as reported in the literature.[34,41] Another metabolite marker, 3-chlorotyrosine, reflects myeloperoxidase-catalyzed oxidation, and its depleted concentration in urine is presumably associated with a low plasma concentration of low-density lipoprotein and triglyceride.[34]

One of the tea components, caffeine, is metabolized in the liver by the cytochrome P450 oxidase enzyme system (specifically, the 1A2 isozyme)[42] into three metabolic dimethylxanthines: paraxanthine, theobromine, and theophylline. Theobromine and theophylline are then metabolized in liver into 1,7-methyluric acid and 1-methylxanthine and subsequently into 1-methyluric acid. These tea metabolites are found in urine samples as both intact tea polyphenols and metabolites of the absorbed polyphenols.

Flavonoids and hydroxycinnamic acids are polyphenolic compounds present in our daily diet in plants and vegetables, as well as in herbal remedies used in herbal medicine.[40] The putative quercetin metabolites, hydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid), were detected in urine. Hippuric acid and hydroxyhippuric acid, the glycine conjugate of benzoic acid, are also detected in urine samples which supports the previous findings that these metabolites are involved in a central metabolic pathway for dietary flavonoids.[43,44] Catechins and their condensed polymers are metabolized into valerolactones and then to phenylpropionic acids, and further to benzoic acids by gut microbes, which are then excreted in urine as hippuric acid after conjugation with glycine.[20,45] The characterization of hepatic and gut microbial biodegradation of plant components as well as the plant-induced alterations in metabolites of symbiotic gut microbes in this study will advance our mechanistic understanding and biomarker discovery for multicomponent botanical agents. The changes of an overwhelming amount of metabolic end points, including changes in our metabolic regulatory pathways and gut microbial-human cometabolism, can be integrated as a systems response to the absorption, disposition, and drug−drug interactions of the tea/dietary intervention system. The integrated approach with the utilization of multivariate statistical tools in this study simultaneously visualizes the intercorrelation between compounds originating from different sources and the metabolic impact of a botanical/drug intervention, which has never before been accessible to the pharmaceutical and nutraceutical industries.

We have shown that, with the aid of a metabonomics and multivariate statistical approach, the bioavailable plant metabolite profile *in vivo* as well as the human metabolic response profile can be identified and characterized simultaneously. A 2 week consumption of polyphenol-rich Pu-erh tea resulted in a significantly altered human metabolic profile and also a wide range of biodegradated tea components. The metabonomics strategy can play an important role in nutritional intervention studies to help unravel the complex interactions between multicomponent nutraceuticals and the human metabolic system. Understanding the metabolic fate of dietary or drug components in a multicomponent intervention will greatly advance the "network pharmacology" strategy and accelerate

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the process of the preclinical study of botanical drug candidates. Furthermore, understanding of the metabolic characteristics of a multicomponent intervention will aid in the clarification of possible toxic or pharmacologically active dietary or drug components, and can also be used for the design of the next generation of drugs to circumvent an undesired metabolic fate of certain drug components.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary methods; information including standard meals, body weight, blood pressure, and bedtime and morning time of participants; identified bioavailable pu-erh tea components; metabolites produced *in vivo* after pu-erh tea intake; altered endogenous metabolites intervened by pu-erh tea ingestion; differential metabolites detected from the metabonome after Pu-erh tea intake (postdose) as compared to predose metabonome; representative base peak intensity (BPI) chromatograms of pu-erh tea and urine at different time points derived from UPLC-QTOFMS analysis; representative total ion current (TIC) chromatograms of pu-erh tea and urine at different time points derived from GC-TOFMS analysis; PCA scores plot obtained from human urine samples at different time points; OPLS-DA scores plots and S-plots of metabonomic comparison among the groups of 24 h, 2 week, and 2 week wash-out after pu-erh tea intake based on the spectral data of (A) UPLC-QTOFMS analysis and (B) GC-TOFMS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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