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ORIGINAL CONTRIBUTION

Identification of urinary metabolites with potential blood pressure-lowering effects in lentil-fed spontaneously hypertensive rats

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Abstract
Purpose Urine samples were obtained from a previously completed study that showed lentil consumption attenuates the increase in blood pressure that occurs over time in spontaneously hypertensive rats (SHRs). The objective of the present study was to compare the metabolite profile of the urine samples from control and lentil-fed SHR in relation to the compounds present in lentils but not in other pulses.
Methods The urine samples were from 17-week-old, male SHR fed semi-purified diet prepared with powder (30 %, w/w) from cooked whole pulses or a pulse-free control diet ($n = 8$/group) for 4 weeks. Pulse powders, control diet and urine samples were extracted using acetonitrile and analyzed by a high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QTOF-MS).
Results Twenty-seven metabolites were significantly different in urine samples from lentil-fed SHR compared to SHR fed control diet, but only 7 were not present in the urine of SHR fed other pulses. Of these metabolites, only citrulline is linked to blood pressure regulation via production of the vasodilator nitric oxide (NO). Several arginine-related compounds that are NO synthase substrates or inhibitors were detected in lentils but not the control diet or other pulse powders.
Conclusions Consumption of lentils increases the availability of arginine and several related compounds that could potentially elevate production of NO and contribute to the blood pressure-lowering effects of lentil-rich diets.

Keywords Hypertension · Metabolomics · Spontaneously hypertensive rat · Lentils

Introduction

Hypertension (systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg) and prehypertension (120 mmHg ≥ SBP ≤ 139 mmHg or 80 mmHg ≥ DBP ≤ 89 mmHg) affect more than 50 % of North Americans [1]. Furthermore, every 20 mmHg increase in SBP or 10 mmHg increase in DBP doubles mortality due to stroke and ischemic heart disease [2]. While several classes of pharmacological agents are capable of reducing hypertension once it has manifested, it has been suggested that the high risk of future hypertension development in persons with prehypertension can be controlled primarily through diet and lifestyle changes [2]. However, it is recognized that improvements in diet may also benefit those persons managing hypertension with pharmaceuticals through synergies that improve drug efficacy, thus enabling a reduction in the dose being employed for blood pressure (BP) control [2]. In fact, a number of bioactive compounds present in food sources have been identified as potential

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antihypertensive agents, including inhibitory peptides, various polyphenolics and polysaccharides [3].

Pulse crops are defined as the edible seeds of leguminous plants, harvested for their dry grain, and excluding those primarily grown for oil or harvested green for consumption [4]. Under this definition, varieties of dried beans, peas, lentils and chickpeas would be considered pulses, whereas soybeans and peanuts would not. Pulses are a nutrient-dense food source that contain high amounts of fiber, protein, minerals, vitamins and variable levels of phytochemicals [5]. A recent meta-analysis [6] has shown that consumption of pulses benefits cardiovascular health in humans by affecting biological processes linked to control of BP. It has been proposed that components of dietary pulses such as peptides, isoflavones and arginine-related compounds affect BP control through the renin–angiotensin system and nitric oxide (NO) production [7].

We recently reported that consumption of whole lentils, but not other pulses (beans, peas, chickpeas), can reduce BP in spontaneously hypertensive rats (SHRs) [8]. Since these results were only obtained with lentil-based diets, it was suggested that in addition to the many compounds present in most pulses with potential important physiological activities, certain nutrients and bioactive compounds present only in lentils may be responsible for the physiological improvements in vascular function. Since metabolomic profiling of urine from normotensive and hypertensive animals has identified several metabolites that associate closely with higher BP [9], it was felt that a similar approach could be used to identify metabolic factors that affect BP in response to dietary intervention. Urine is one of the most effective biological fluids for metabolomics studies because it is considered to be noninvasive compared to blood, and urine can provide additional information regarding the efficiency of renal filtration. As well, it can be used to identify the metabolites of molecules absorbed from food and thus provides information useful for pharmacokinetic analysis. In addition, metabolomic studies can lead to the development of noninvasive assays suitable for monitoring consumption patterns and bioactive turnover. Thus, the objective of the present study was to compare the metabolite profile of the urine samples from control and lentil-fed SHR in relation to the compounds present in lentils and determine whether any of the compounds in lentils and/or their urinary metabolites associate with known mechanisms for BP-lowering. Furthermore, it was anticipated that this approach would assist with the formulation of a hypothesis capable of explaining the BP-lowering that was observed with lentil consumption and could be tested in a subsequent experiment.

Materials and methods

Chemicals

HPLC-grade acetonitrile, methanol, spectroscopic-grade formic acid, D-norvaline and L-citrulline were purchased from Sigma–Aldrich Ltd (Oakville, ON, Canada). L-homocitrulline was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). N^{ω}-hydroxy-L-arginine monoacetate was purchased from Enzo Life Sciences (NY, USA). Double-distilled water (*dd*H_2O) was collected at 18 Ω from a Barnstead E-Pure system (Dubuque, Iowa, USA). The ESI low concentration tuning mix and API-TOF reference mass solution were purchased from Agilent Technologies (Mississauga, ON, Canada). CDC Greenland green lentils and CDC Imax CL red lentils were from Simpson Seeds (Moose Jaw, SK, Canada).

Extraction of pulse compounds

Subsamples (~150 mg) of previously cooked and freeze-dried pulse powders (lentils: red and green; beans: pinto, navy, red kidney, black; peas: yellow, green; chickpeas) used in the diets of the previous study, as well as the control diet, were spiked with 10 µL 2-aminopentanoic acid (0.03 mg/mL) as an internal standard and extracted in triplicate with 2 mL of methanol/water (3:2, v/v) by immersion in an ultrasonic bath (Branson 1200 sonicator, Shelton, CT, USA) for 40 min. Subsequently, the samples were vigorously vortexed (10 s) and centrifuged (14,000 g for 10 min). A subsample from the resulting supernatant (1 mL) was dried in a Vacufuge Plus, and the dry residue was redissolved in 200 µL of 1:4 acetonitrile:*dd*H_2O as explained for rat urine extracts.

Extraction of urinary metabolites

Urine was collected during the course of an animal study described in detail elsewhere [8]. Briefly, the study compared the effects of dietary interventions on BP in 17-week-old, male SHR (n = 8/group) fed semi-purified diet containing pulse powder (30 %, w/w) for 4 weeks. Control SHR was maintained on a pulse-free control diet for the same time period. The details of this intervention and the diet formulations have already been published [8]. These experiments were carried out in accordance with proper animal care and experimentation as outlined by the Canadian Council on Animal Care and a protocol approved by the University of Manitoba Animal Care Committee. Urine was collected during the final week of dietary intervention, with animals placed individually in metabolic cages for 12 h with water but no feed, and stored at –80 °C.

An aliquot of urine was thawed at room temperature, vortexed (3 s) and centrifuged for at 4 °C for 5 min at 10,000*g*. Two subsamples (50 µL) were subsequently transferred into disposable test tubes for osmolarity measurements (Precision System, Inc. for Osmettes) using an osmometer (5004 Micro-Osmette, Freezing Point Osmometer, MA, USA). The osmometer was first calibrated for the 0–2 mOsm range using osmometry standard solutions of 100 and 500 mOsm/kg H_2O (Precision System Inc., MA, USA). The calibration was tested using Control 290 mOsm/kg H_2O solution (Cat: 2202).

A separate aliquot of urine was thawed at room temperature, vortexed (30 s) and placed in 250 µL microfuge tubes containing 500 µL of acetonitrile, while blank samples were prepared with 250 µL of deionized water instead of urine. All urine samples were spiked with 10 µL of a 2-aminopentanoic acid solution (0.03 mg/mL *dd*H_2O) as an internal standard. The mixture was vortexed (30 s) and centrifuged at 13,000*g* at room temperature for 20 min. Supernatants were transferred to new microfuge tubes and dried under vacuum in a Vacufuge Plus (Eppendorf, Settings V-HV) at 45 °C, and kept at −20 °C prior to reconstitution for LC-QTOF-MS analysis. Dried samples were reconstituted in 200 µL of 1:4 acetonitrile: *dd*H_2O using glass insert and brown LC vials. This 1:4 acetonitrile:*dd*H_2O mixture was used as a blank throughout this study. All extractions were performed in triplicate.

Quality control samples for metabolomics

Five quality control mixtures were made by pooling (250 µL) of urine (randomly chosen from two rats in each group) and were analyzed in a random manner among all other samples.

Liquid chromatography

A 1260 Rapid Resolution system (Agilent Technologies, Santa Clara, CA, USA) containing a binary pump and degasser, well-plate autosampler with thermostat (1290), and thermostatted column compartment was used for all analyses. Chromatographic separations of the samples were performed on an Agilent ZORBAX SB-Aq column 2.1 mm × 100 mm, 1.8 µm, at a column temperature of 45 °C. The mobile phases A and B were water and acetonitrile with 0.1 % formic acid, respectively. The gradient duration was 10 min with 0–6 min 2 % B; 6–8.50 min 60 % B; 8.50–10 min 2 % B. A post-run time of 2 min was used prior to new injections. In order to minimize the carryover of samples during successive injections, the injection needle was washed with the mobile phase in two separate vials (5 washings per vial) before each injection. The autosampler temperature was maintained at 6 °C. The flow rate was 0.7 mL/min. Aliquots (1 µL) of urine and lentil extracts were injected for individual analysis.

Mass spectrometry (MS) and MSMS analyses

MS was performed on an Agilent 6538 Q-TOF mass spectrometer equipped with a dual electrospray ionization source (ESI) in positive mode. The capillary voltage, the fragmentor, the skimmer and the OCT 1 RFVpp were set to 4,000, 80, 50 and 750 V, respectively. The drying gas (N_2) was set to 11 L/min at 300 °C and the nebulizer at 50 psig. Spectra were acquired over the 50–1,200 m/z range. Reference masses of 121.0508 and 922.0097 (ESI+) and 112.9860 and 1033.9880 (ESI−) were used for all runs. Targeted MSMS analysis of the pure standards and urine and pulse extracts was performed to identify the potential biomarkers. As part of the MassHunter Software, the collision energy was applied by setting an appropriate equation having a slope value of 5 and offset value of 2.5. A full range mass scan from 50 to 1200 m/z with an extended dynamic range of 2 GHz standardized at 3200 was applied. The data acquisition rate was maintained at 3 spectra/s at a time frame of 333.3 ms/spectra with a transient/spectrum ratio of 1932.

The semi-quantification of the selected significant compounds in pulse extracts was performed using the ratio of the area of the base peak obtained for selected compounds to that obtained for the 0.3 µg of added internal standard (2-aminopentanoic acid; [M + H] = 118.0863 or [M − H] = 116.0715 in ESI+ and ESI− modes, respectively).

Pooled quality control (QC) samples were used to ensure the suitability of the method for both rat groups analyzed in this study. In addition to running the pooled QC samples with all other urine samples, 15 injections of the same urine sample (a mixture from individual rats) were carried out to validate the extraction and the LC–MS method. The Molecular Feature Extraction (MFE) was applied to all 10 replicates, and features with abundance >5000 were extracted. Average values for RT and m/z values of each feature were calculated.

Data processing and statistical analysis

Unbiased processing of the entire metabolomics dataset for the pulse extracts and the urine samples was performed by Agilent MassHunter Qualitative (MHQ, B.07) and Mass Profiler Professional (MPP, 12.6.1) as previously described [10, 11] and summarized in Table 1. The raw data files were first acquired and stored as "*.d" files using an Agilent MassHunter Acquisition software (B.07) ready to be processed in MHQ. The Molecular Feature Extraction (MFE), a naïf extraction procedure, was the first algorithm applied

Table 1 Summary of the metabolomics workflow

Step 1: Metabolomics of Rat Urine (LC-QTOF-MS and MHQ 7.01)
Non-targeted analysis of all urine samples by LC-QTOF-MS in ESI positive and negative modes
Molecular Feature Extraction (MFE) algorithm was used to extract all detectable compounds
Find by Ion algorithm was used to remove false ± compounds
Partial least square discrimination algorithm on the final filtered entity list containing 1235 and 1303 entities, in ESI+ and ESI− modes, respectively (Fig. 1a, b), and generation of Lorenz curves (Supplementary Figures S1–S4)
Step 2: Statistical Analysis (MPP 12.6.1)
Unpaired *t* tests ($P < 0.01$) followed by a robust multiple testing correction of p values (Bonferroni FWER) comparing metabolites in urine from SHR control versus SHR lentil-fed [27 metabolites with significant changes (Table 2)]
Step 3: Search by Formula Algorithm (MHQ 7.01)
Search by Formula algorithm was used to determine whether the 27 metabolites in urine from SHR lentil were present in urine samples of SHR fed other pulses. Seven metabolites were present primarily in urine of SHR lentil-fed compared to the other pulses (Table 3)
Step 4: Pathway Analysis
Identification of biochemical pathways for the metabolites present primarily in urine of SHR lentil-fed and their potential roles in regulation of blood pressure. Of the 7 metabolites, only 4 were known compounds, and of these only citrulline had possible links to blood pressure regulation through arginine and nitric oxide.
Step 5: Analysis of Pulse Extracts (LC-QTOF-MS and MHQ 7.01)
Analysis of pulse extracts and control diet by LC-QTOF-MS in ESI+ mode (Table 4). Identification of arginine and its related compounds as potential bioactive compounds in lentil powder

to the total ion chromatogram (TIC) files. The MFE parameters were set to allow the extraction of detected features with absolute abundances ≥4000 counts providing information regarding $[M + H]^+$, isotopes and their corresponding Na^+ adducts. The resulting extracted ions were treated as single features for which potential formula was generated. The collected information summarizing retention time (tR), exact masses and ion abundances were converted into compound exchange format ("*.cef") and were exported to MPP for further subsequent comparative and statistical analyses. All data were transformed to Log 2 scale. Using alignment and normalization procedures (osmotic pressure values were entered as external scalar values to correct for potential differences in diuresis between animals), individual "*.cef" files were binned and combined to generate new "*.cef" files. These new files were reopened in MHQ for further data mining procedure using a "Find by ion" algorithm. This targeted feature algorithm helped with minimizing the false positive and negative features found by the MFE procedure. A second series of individual "*.cef" files were created from the original individual "*.d" files and exported into MPP for statistical and differential analysis. A frequency filtration was used to accept features that were detected in at least one condition. This filtration step was employed to ensure elimination of the potential feature extraction artifacts.

Other MPP filtering procedures such as number of detected ions (set to "2") and charge states (set to "all charge states permitted") were also applied. The retention time compound alignment parameters were set to 0.15 min with a mass tolerance of 2.0 mDa.

The final processed lists contained 1235 and 1303 compounds in ESI+ and ESI− modes, respectively. The filtration step in the data processing ensured that the final list included metabolites that were present in at least 50 % of the urine samples from the control or the lentil-fed groups. An unpaired *t* test ($P < 0.01$) followed by a multiple testing correction of the final *P v*alues (Bonferroni FWER) was used to compare urinary metabolites found in control versus lentil-fed SHR. These data were used to create a new "*.cef" file which was examined with the "Search by Formula" algorithm within MPP (12.6.1) to determine whether any of these metabolites were present in the urine samples from SHR fed the other pulses. In addition, a prediction model was built using the partial least square discrimination (PLSD) algorithm (MPP 12.6.1). The following validation parameters were used: number of components (4), validation type (N-fold), number of folds (3) and number of repeats (10). The results generated by PLSD were used to examine the predicted label and confidence value for each individual sample ($n = 16$). Lorenz curves were displayed for each of the two class fraction (two diet treatments) to visualize the ordering of this measure for each class.

Results

The LC-QTOF-MS methodology employed in our study was capable of detecting more than 10,000 compounds in the rat urine samples. There was a clear separation between the two groups (control vs lentil-fed SHR) when the partial

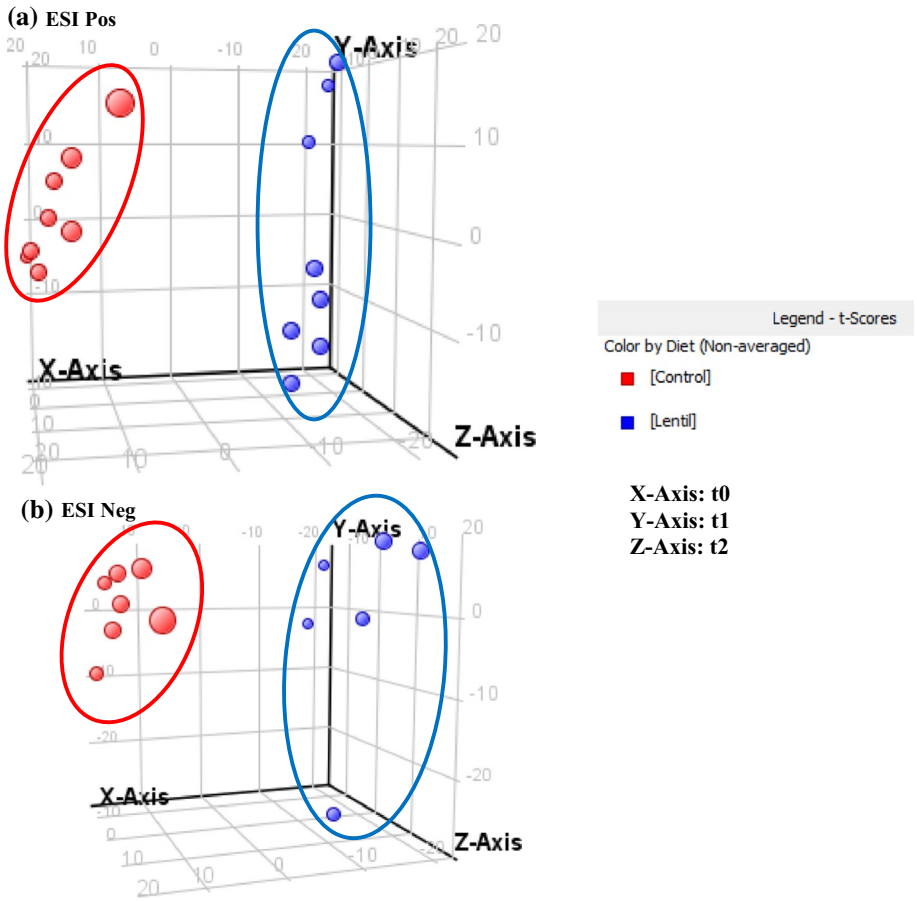

Fig. 1 An overview of the t-scores from partial least squares discrimination (PLSD) analyses of rat urine metabolites in SHR lentil-fed and SHR control run in ESI positive (**a**) and ESI negative (**b**) modes. Each *circle* represents an individual rat with $n = 8$ rats/group

least square discrimination (PLSD) algorithm was applied to the final filtered entity list containing 1235 entities in ESI+ mode (Fig. 1a) and 1303 compounds in ESI− mode (Fig. 1b). These figures showed a clear separation of SHR rats belonging to two different diet groups based on the profile of urinary metabolites in both ESI+ and ESI− modes. A closer examination of these clusters suggested a tighter distribution of individual rats (each circle on the graph) within the control group compared to the lentil-fed groups. This might suggest a higher variability in urinary metabolites among SHR rats fed the lentil diet. These graphs also provide information regarding the overall consistency of the experimental work including the dietary intervention in SHR, urine collection and extraction and metabolomics analyses. The accuracy of the prediction using this algorithm was 93 and 84 % for ESI+ and ESI− modes, respectively. The Lorenz curves generated from the data verified the accuracy of the PLSD predictions as indicated by the presence of the 8 samples belonging to one group forming a single line with linear slope from 0 to 1 on the y-axis, and the 8 samples from the other group distinctly located on the flat line (Supplemental Fig. S1–4). Each of the Lorenz curves showed a break in the slope at the midpoint of the line, thus confirming that there was a clear separation in the metabolite composition of all 8 samples belonging to a particular treatment group.

Unpaired *t* tests revealed 27 urinary metabolites were significantly different ($P < 0.01$) between SHR fed control versus lentil diet (Table 2). The identity of these metabolites was determined using MassHunter ID browser program and MassHunter Qualitative software to search the Metlin database (contains approximately 79,000 metabolites, 168,000 peptides and 31,000 lipids) and subsequently confirmed by comparing the MS/MS spectra of their respective pure standards (when available). These data are presented in descending order of fold change (lentil versus control) for each metabolite (reported as both Log 2 values and absolute values of ion abundance), as well as their m/z values, ESI polarity, the generated formula, and their putative associated biochemical pathway(s). Plotting these data as a heatmap showed consistency in the presence of the various metabolites among individual rats fed either control or lentil diet (Fig. 2). There was a high degree of consistency for the listed compounds, particularly in the SHR fed control diet. Interestingly, the urinary concentrations of citrulline and homocitrulline were highly abundant in 7 of the 8 SHR rats fed the lentil diet, whereas rat number 5 had urinary concentrations of citrulline and homocitrulline

Table 2 Metabolites showing significant change (unpaired *t* tests; $P < 0.01$) in urine of spontaneously hypertensive rats (SHR) fed control versus lentil-based diets as detected using electrospray ionization (ESI) in both positive (+ve) and negative (−ve) modes

N	Metabolite	Log 2* (FC)	Fold change	P (Corr)*	Regulation (Lentil *vs.* Control)	m/z	ESI* polarity	tR* (min)	Formula	Associated pathway
1	Citrulline	15.50	46,341	5.80E−04	Up	176.1027	+ve	2.18	$C_6H_{13}N_3O_3$	Urea cycle and arginine metabolism
2	Homocitrulline	15.00	32,768	5.80E−04	Up	190.1181	+ve	1.75	$C_7H_{15}N_3O_3$	Urea cycle and arginine metabolism
3	Unknown 1	13.70	13,308	3.20E−07	Up	285.0635	−ve	3.76	$C_{12}H_{14}O_8$	–
4	Unknown 2	13.30	10,086	1.60E−03	Up	270.0541	+ve	5.16	$C_{10}H_{11}N_3O_4S$	–
5	Unknown 3	13.00	8192	5.10E−04	Up	438.9803	+ve	2.23	$C_8H_{18}O_{14}P_2$	–
6	2-Oxoarginine	12.60	6208	3.40E−03	Up	174.0874	+ve	1.83	$C_6H_{11}N_3O_3$	Arginine metabolism
7	Pyridoxamine	12.40	5405	2.30E−03	Up	167.0814	−ve	3.25	$C_8H_{12}N_2O_2$	Vit B6
8	Unknown 4	12.20	4705	2.40E−03	Up	204.9813	−ve	4.14	$C_8H_8Cl_2O_2$	–
9	Ala–Ala	12.10	4390	5.70E−03	Up	159.0771	−ve	4.07	$C_6H_{12}N_2O_3$	Peptide
10	Ile-Ala-Val	11.30	2521	3.40E−03	Up	302.2074	+ve	3.63	$C_{14}H_{27}N_3O_4$	Peptide
11	Unknown 5	10.50	1448	3.70E−23	Up	247.1441	+ve	8.76	C_7H_9NO	–
12	5-Aminopentanamide	8.50	362	7.10E−03	Up	115.0872	−ve	3.99	$C_5H_{12}N_2O$	Lysine metabolism
13	Gly-Cys	4.30	20	9.00E−03	Up	177.0354	−ve	2.36	$C_5H_{10}N_2O_3S$	Peptide
14	Pro-Ser	2.10	4	4.90E−04	Up	203.1027	+ve	3.56	$C_8H_{14}N_2O_4$	Peptide
15	4-Guanidinobutanoic acid	1.40	3	6.30E−04	Up	146.0924	+ve	1.92	$C_5H_{11}N_3O_2$	Arginine and proline metabolism
16	20-Hydroxy-PGD2	0.80	2	3.60E−03	Down	407.1844[£]	+ve	8.67	$C_{20}H_{32}O_6$	Prostaglandins
17	Pro-Phe	1.80	3	1.30E−03	Down	263.1393	+ve	8.74	$C_{14}H_{18}N_2O_3$	Peptide
18	1-Methylhistamine	1.90	4	2.30E−03	Down	126.1026	+ve	1.37	$C_6H_{11}N_3$	Histidine metabolism
19	Unknown 8	2.10	4	1.60E−03	Down	303.0273	+ve	1.23	C_7H_5NOS	–
20	Gluconasturtiin	2.30	5	7.50E−04	Down	864.1543[£££]	+ve	1.53	$C_{15}H_{21}NO_9S_2$	Glucosinolate from phenylalanine, tryptophan and tyrosine biosynthesis
21	N_1-Acetylspermidine	2.30	5	1.20E−04	Down	188.1757	+ve	1.42	$C_9H_{21}N_3O$	Endogenous metabolite
22	Unknown 7	2.40	5	3.80E−03	Down	739.1335	+ve	1.55	$C_{35}H_{30}O_{18}$	–
23	Unknown 6	2.60	6	2.10E−03	Down	489.1101	+ve	1.52	$C_{18}H_{32}O_7S_4$	–
24	Selenocysteine	3.60	12	3.20E−04	Down	364.9042[£]	+ve	1.46	$C_3H_7NO_2Se$	Amino acids
25	3,4-Dihydroxyphenyl glycol	4.00	16	2.60E−02	Down	171.0648	+ve	8.82	$C_8H_{10}O_4$	Tyrosine metabolism
26	Alpha-aspartyl-hydroxyproline	5.60	49	1.40E−03	Down	245.0768	−ve	2.53	$C_9H_{14}N_2O_6$	Peptide
27	Lysine	15.70	53232	6.30E−06	Down	147.1126	+ve	1.30	$C_6H_{14}N_2O_2$	Lysine metabolism

* Log 2 (FC), Log 2 values for fold changes; P (Corr), multiple testing correction using Bonferroni FWER; ESI, electrospray ionization mode; tR, retention time (min)

[£] [M + K]+

[£££] [2 M + H]+

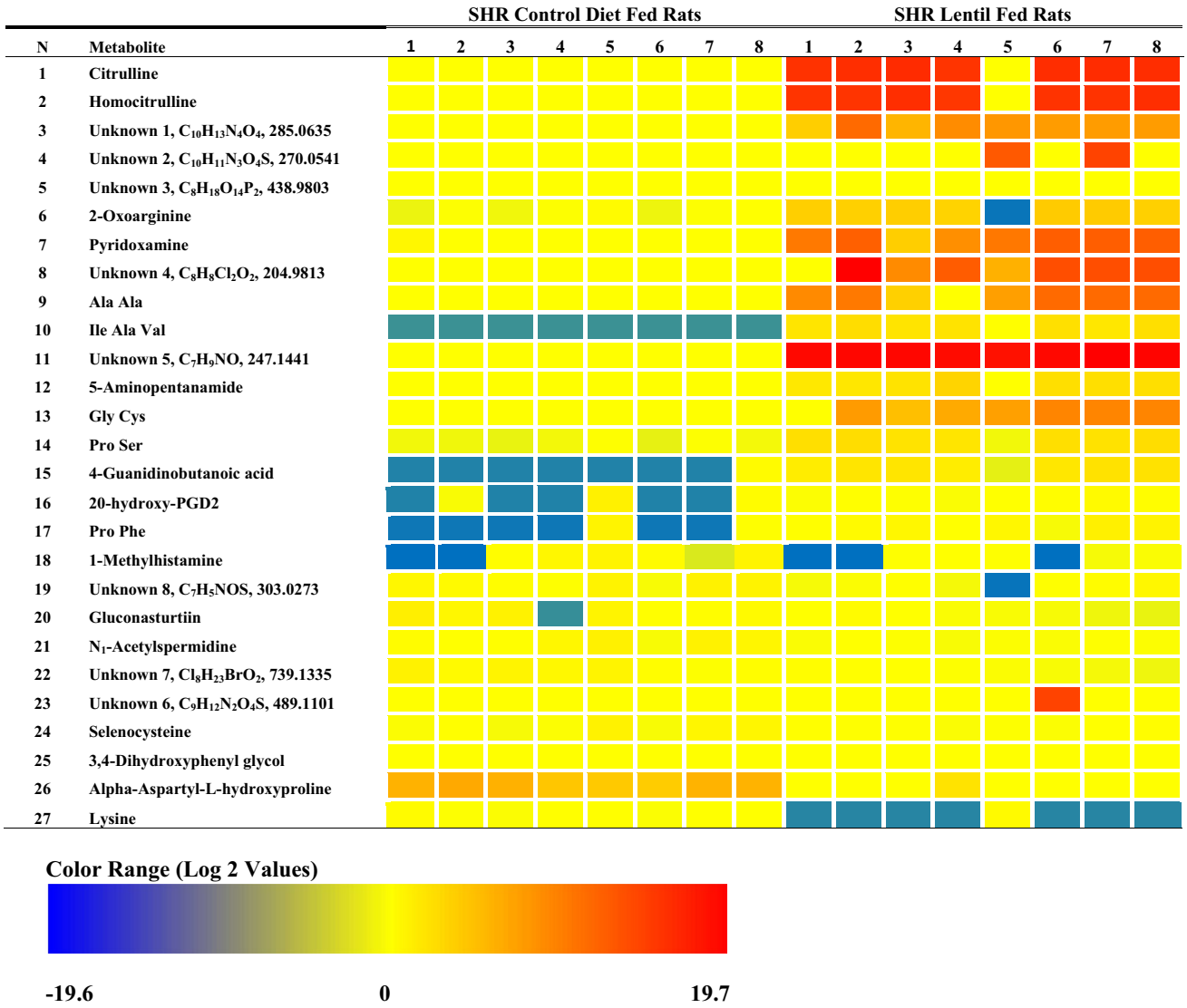

Fig. 2 A heatmap of the clustering hierarchical results for the 27 metabolites that were different in urine from SHR lentil-fed versus SHR control rats. Each *row* represents a metabolite, and each *square within a row* represents an individual rat. All values are Log 2-normalized values of detected abundance for each metabolite. The *color scale* represents relative abundance of going from high (*red*) to low (*blue*)

similar to those obtained in control SHR. Given that there was some variation in other compounds in rat 5 and other rats [for example, Unknown 2 (rats 5 and 7), 2-oxoarginine (rat 5), Unknown 4 and Gly Cys (rat 1), 1-methylhistamine (rats 1, 2, 6), Unknown 8 and lysine (rat 5) and Unknown 6 (rat 6), this likely represents biological and physiological variations among the rats and not variation due to sample preparation and/or metabolomics analysis.

To determine whether the urinary metabolites listed in Table 2 were unique to lentil-fed SHR, the "Find by Formula" algorithm of the MPP software was used to search urine samples collected from SHR rats fed other pulses for the same 27 metabolites (Table 3). The abundance of the metabolites in the urine samples from SHR fed beans, peas or chickpeas is presented as a percentage relative to the amount present in urine from lentil-fed SHR. In total, only 7 metabolites (citrulline, Unknown 6, Ala–Ala, pyridoxamine, Unknown 4, Unknown 1, 5-aminopentanamide) were present primarily in urine samples obtained from the lentil-fed group, as indicated by the fact <5 % was present in samples from the bean, pea and chickpea fed groups. The presence of a metabolite in the urine of rats fed at least one pulse type was deemed sufficient to exclude it from the list of compounds that could potentially contribute to the actions of the lentil diet on BP, and so the remaining 20 entities were eliminated from further consideration.

Table 3 Proportion of metabolites in the urine of SHR fed beans, peas or chickpeas compared to SHR-fed lentils

N	Compound	Beans	Peas	Chickpeas
		% of lentils		
1	Citrulline	0.0	0.0	0.1
2	Homocitrulline	0.0	361	0.0
3	Unknown 1, $C_{12}H_{14}O_8$, 285.0635	0.0	0.0	0.0
4	Unknown 2, $C_{10}H_{11}N_3O_4S$, 270.0541	450,648	1962	112,610
5	Unknown 3, $C_8H_{18}O_{14}P_2$, 438.9803	440	7803	461
6	2-Oxoarginine	19.5	32.4	0.0
7	Pyridoxamine	0.0	0.9	0.5
8	Unknown 4, $C_8H_8Cl_2O_2$, 204.9813	0.0	0.0	0.1
9	Ala Ala	0.2	3.4	0.0
10	Ile Ala Val	1.7	47.8	0.0
11	Unknown 5, C_7H_9NO, 247.1441	0.0	0.0	64.5
12	5-Aminopentanamide	0.1	0.3	0.2
13	Gly Cys	367	105	413
14	Pro Ser	20.0	19.2	2.7
15	4-Guanidinobutanoic acid	41.6	43.5	2.6
16	20-Hydroxy-PGD2	22.0	119	20.1
17	Pro Phe	121	4.1	2.3
18	1-Methylhistamine	13.4	110	602
19	Unknown 8, C_7H_5NOS, 303.0273	0.0	443	107
20	Gluconasturtiin	22.3	133	162
21	N_1-Acetylspermidine	92.5	119	100
22	Unknown 7, $Cl_8H_{23}BrO_2$, 739.1335	4.9	121	151
23	Unknown 6, $C_9H_{12}N_2O_4S$, 489.1101	22.0	22.0	22.0
24	Selenocysteine	112	112	108
25	3,4-Dihydroxyphenyl glycol	362	100	100
26	Alpha-aspartyl-L-hydroxyproline	14,513	25,330	60,480
27	Lysine	239,0376	300,5823	553,895

Examination of the biochemical pathways associated with 3 of the known lentil-specific compounds showed links to vitamin B6 (pyridoxamine), lysine metabolism (5-aminopentanamide) and urea cycle/arginine metabolism (citrulline), while the dipeptide Ala–Ala has not been assigned to any pathway. Of these, only citrulline is linked to BP through its close relationship with the vasodilator NO. For this reason, we decided to examine lentils for compounds associated with citrulline production.

Analysis of the pulse extracts confirmed that green and red lentils contained substantial amounts of L-arginine (286 and 552 µg/100 g, respectively) relative to the control diet (Table 4). Furthermore, the abundance of this amino acid in lentils was due to the presence of free amino acids and not due to the arginine content of dietary protein, which was identical in both the lentil and control diet formulations. In addition to L-arginine, two related compounds were detected in lentils: symmetric dimethylarginine (SDMA) and $C_6H_{14}N_4O_3$ (Table 4). The MS spectra obtained for $C_6H_{14}N_4O_3$ did not allow for unequivocal identification of this compound. In fact, the Metlin database identified four isobaric arginine-related compounds, beta-hydroxyarginine, L-hydroxyarginine, gamma hydroxyl arginine and N^{ω}-hydroxy-L-arginine as potential candidates with similar m/z (191.1137), formula and identification score (~97 %). However, none of these hydroxyl arginines are involved in reactions where citrulline is formed except for N^{ω}-hydroxy-L-arginine. It should be noted that an MSMS breakdown of N^{ω}-hydroxy-L-arginine in ESI+ (with a collision energy of 12.1 v) into m/z 174.1063 = [M–OH + H], 157.0602 = [M–OH–NH_3 + H] and 111.0547 = [M–OH–NH_3–H_2O + H] (Fig. 3) supports the view that this compound is responsible for the $C_6H_{14}N_4O_3$ detected in this analysis. Thus, the presence of arginine and/or an arginine-related compound in lentils but not in control diet and in other pulses (Table 3) could have accounted for the elevated urinary levels of citrulline in SHR fed the lentil diet (Table 2) via an enzymatic reaction catalyzed by nitric oxide synthase (NOS).

Discussion

In this study, non-targeted metabolomics was used to identify compounds that associate with known mechanisms for BP-lowering and may be responsible for the ability of a lentil-rich diet to block the progressive increase in BP that occurs as SHR become older (systolic BP: +26 mmHg for SHR on lentil-free control diet vs +1 mmHg for SHR on lentil diet) [8]. It is important to note that other pulses used in our previous study (beans, peas, chickpeas or a mixture with equal amounts of all the pulses) failed to attenuate the SBP in our SHR rat model indicating that only lentils had the capacity to affect BP [8]. Metabolomics was used in this study as an approach that can aid in the generation of new hypotheses that may explain the BP-lowering effects observed in SHR fed a lentil diet. The outcome of the current metabolomic analysis was the identification of 7 compounds that are uniquely present in the urine of animals fed the lentil diet but not the other pulse-containing or control diets. Two of these compounds present in lentils (SDMA and N^{ω}-hydroxy-L-arginine) were recognized as being capable of modulating NOS activity. This observation, coupled with the presence of urinary citrulline, provided

Table 4 Arginine-related compounds present in extracts of the control diet and pulse powders (μg/100 g freeze-dried powder)[a]

Compound[b]	m/z (ESI +)	Control diet	Pinto beans	Navy beans	Red kidney beans	Black beans	Chickpea	Yellow pea	Green pea	Green lentil	Red lentil
L-Arginine	175.1187	ND[c]	894	1312	310	742	1062	3141	513	286	552
Symmetric dimethylarginine	203.1502	ND	ND	ND	ND	ND	135	493	70	143	171
N^{ω}-hydroxy-L-arginine	191.1137	ND	ND	ND	ND	ND	ND	ND	ND	1784	1241

[a] The concentration for each metabolite was calculated based on addition of 0.3 μg of internal standard (D-norvaline); [M + H]+= 118.0863) to freeze-dried powders (150 mg) prior to extraction; average of triplicate analysis

[b] Compounds were identified by MS spectra using LC–MS-QTOF

[c] ND: not detected or abundance of the mass was below the cutoff threshold level

sufficient evidence to hypothesize that these arginine-related compounds are associated with the vasodilator NO (and formation of citrulline), and this could explain how lentils reduced the BP of SHRs. This is a hypothesis that can and will be tested in future animal and human studies. Furthermore, it was determined that while lentils have an abundance of L-arginine, a key precursor of the vasodilator NO, the high levels of L-arginine in other pulses suggests it is likely not responsible for the BP effects of lentils.

The non-targeted approach utilized in this study required several choices to be made as the analysis progressed. A total of 2538 compounds were detected in the samples, but *t* tests determined there were only 27 that were significantly different in the urine samples taken from the lentil-fed and control diet groups. Subsequently, it was shown that 20 of these compounds were also present in urine samples from SHR fed beans, peas and chickpeas, thus indicating only 7 compounds specifically associated with lentil feeding. However, three of these compounds are unknown, and thus, further assessment of their potential contribution to BP regulation was not possible. While some peptides can inhibit angiotensin converting enzyme and thus lower BP by reducing circulating angiotensin II levels, the dipeptide Ala–Ala does not conform with the structural features necessary for this activity [12]. However, it has been reported to exhibit antimicrobial effects, but only when in the D conformation [13]. Similarly, several reports have shown that pyridoxamine, which is a form of vitamin B6, does not influence BP [14, 15]. The biological relevance of 5-aminopentanamide (also known as 5-aminovaleramide), a cellular breakdown product of lysine, has not been examined. Furthermore, no changes in its levels in any biological tissue or fluid have been reported. However, its presence in the urine may explain the low levels of lysine present in the urine of SHR fed the lentil diet. While differences in diet quality may be a factor that affects urinary lysine content [16], there is no evidence that lysine or its metabolites are able to affect BP. This is in contrast to carboxymethyl-lysine, which may indicate arterial stiffness when present at elevated levels in the circulation [17]. However, carboxymethyl-lysine is excreted unchanged and so urinary lysine *per se* cannot be considered an indicator of altered arterial function [18].

Citrulline is closely linked to arginine metabolism via two distinct pathways. In the urea cycle, arginine is converted to ornithine by arginase, and further metabolized to citrulline via ornithine carbamoyl transferase. As well, citrulline is a direct product of the reaction catalyzed by NOS that produces NO from arginine. Since NO is a vasodilator produced by the endothelial cells of blood vessels and has a significant role in BP control, the high levels of L-arginine in the lentil diet compared to the control diet may lead to increased NO production through greater substrate

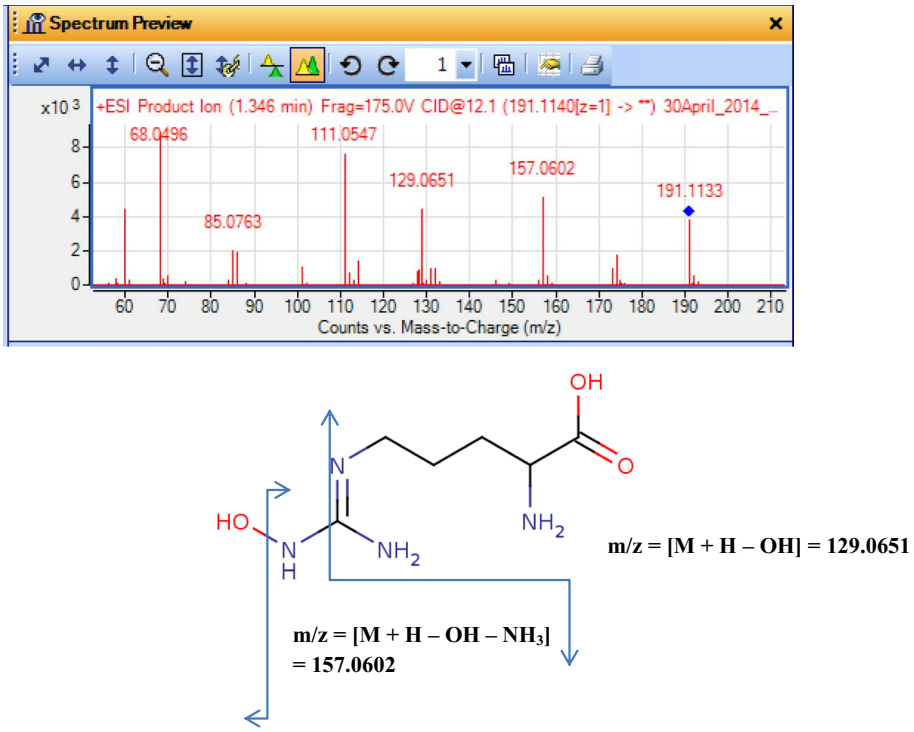

Fig. 3 MSMS spectra of $N^{(\omega)}$-hydroxy-L-arginine [M + H] = 191.1133 in lentil extract by LC-QTOF in MSMS mode

availability. This could explain why lentil consumption reduces BP as we have previously reported [8]. However, the available evidence indicates that increasing L-arginine via supplementation may not increase activity of NOS [19], and it may actually boost mortality in certain conditions [20]. This discrepancy between L-arginine levels and NO production has been noted previously and has been termed the arginine paradox [21]. In general, the L-arginine content of endothelial cells is already sufficiently high to saturate endothelial NOS (eNOS). As a result, high exogenous levels of L-arginine are typically unable to promote greater NO production [22]. Furthermore, since all of the pulses contained L-arginine, it is unlikely that this compound explains the lowering of blood pressure only in the group fed the lentil diet.

Although L-arginine is the primary source for production of the BP-lowering NO, other arginine-related compounds have been reported to affect BP, and two of these compounds were detected in lentils but not other pulses (Table 4). SDMA has been shown to reduce NO production by endothelial cells through inhibition of eNOS [23]. SDMA is an effective competitive inhibitor of L-arginine, capable of interfering with NOS function in the presence of a 100-fold excess of L-arginine [23]. However, the high intracellular levels of L-arginine may be sufficient to outcompete SDMA. Furthermore, it is not known how effectively SDMA is transported into cells. The fact that lentil consumption does not increase BP suggests that the SDMA content is not high enough to have a physiological effect.

Although $N^{(\omega)}$-hydroxy-L-arginine is a less efficient substrate of eNOS than L-arginine [24–26], it has been shown to cause endothelium-independent vascular relaxation [27] through its ability to promote the second step of the catalytic reaction catalyzed by NOS [25]. Based on the amount in the diet (~1.7 and 1.2 mg/100 g of freeze-dried green and red lentil powder, respectively) and average feed consumption, the rats ingested ~0.2 mg of $N^{(\omega)}$-hydroxy-L-arginine daily. However, whether absorption of this compound from a food is sufficient to affect BP to the degree we have observed [8] remains unclear, especially since this compound has not been tested in a relevant animal model.

While a number of reports have indicated lentils can lower BP by interfering with elements of the renin–angiotensin system [28, 29], one of the key physiological mechanisms used to regulate BP, the outcome of this metabolomics study has been the identification of compounds that may instead modulate the production of NO. However, to determine whether NO synthesis is affected by compounds absorbed from lentils, it would be necessary to determine the level of NO in the circulation. Since our LC-QTOF-MS system was set to scan the m/z range of 50-1,000, NO with a mass of 31.0058 could not be detected. The same limitation applies to its products, nitrate and nitrite. As a result, addressing this concept experimentally will be challenging because of this inability to measure NO directly. Therefore, monitoring the vasodilatory effects due to NO will be important for future studies that intend to examine the utility of using lentils as a means of controlling BP in humans.

Biological fluids, such as urine samples, are valuable sources for metabolomics research, since they contain metabolites filtered from the blood by the kidneys and also secreted by tubular epithelia. Sampling urine would be advantageous from a clinical standpoint because it is noninvasive and can be sampled at regular intervals without harm to the patient. Analyzing urine samples using a non-targeted approach provides a complete overview of urinary metabolites, allowing them to be separated upon further analysis to determine diet-specific changes. This approach is beneficial with respect to locating potential metabolites of foods and physiological systems affected by dietary intervention, providing evidence for further hypothesis generation. In the current study, our metabolomics approach was non-targeted and a database of >250,000 endogenous metabolites was employed for both urine samples and pulse extracts. However, no single metabolomics method can detect all existing metabolites. Furthermore, it is possible that a metabolite, and not a native compound in the lentils, may be responsible for the BP-lowering effects or that the positive effects of lentils may be due to the synergistic actions of several compounds.

Conclusions

The strength of this study was its use of a non-targeted urinary metabolomics approach to survey which metabolic processes linked to known BP-lowering mechanisms were altered by a food intervention. This technique made it possible to monitor hundreds of compounds in relation to the composition of the lentils as well as the urinary compounds excreted after metabolism of the compounds by ingesting lentils. The non-targeted metabolomics approach was a valuable tool in the generation of a new hypothesis that links lentil consumption to NO production, and this can be experimentally tested in future animal and/or human studies. Specifically, the presence of certain metabolites (e.g., citrulline) in urine provides evidence for a potential role of arginine-related compounds in lentils for stimulating NO-dependent vasodilation in SHR rats. Consequently, L-arginine and/or several related compounds may potentially contribute to the BP-lowering effects seen with the lentil diet in the SHR model. The results we have obtained can now be used to guide additional experimentation to determine the degree by which these compounds may influence the BP-lowering potential of a lentil containing diet.

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