Original Article

Investigations into Biochemical Changes of Genetic Hypertensive Rats Using ^1H Nuclear Magnetic Resonance-Based Metabonomics

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Genetically hypertensive rats provide a simple and accessible model for studying essential hypertension, which is a polygenic, heterogenous and multifactorial disease. Their genetic and metabolic features are of great interest because they may provide insight into the pathophysiological processes underlying essential hypertension. We have investigated the genetic influence on metabolic balance and metabolite excretion patterns in stroke-prone spontaneously hypertensive rats (SHRSP) with established hypertension using ^1H NMR-based metabonomics. Urinary metabolite profiles for SHRSP and their age-matched normotensive controls, Wistar Kyoto rats, were acquired using ^1H NMR spectroscopy. Principal components analysis was applied to these complex NMR data to facilitate differentiation and determine metabolic differences between urine samples collected from the hypertensive and normotensive rats. Consequently, it was possible to distinguish urine samples between the two strains in the principal components scores plot. The loadings plot showed that taurine, creatine and some unidentified metabolites resonating at around δ 2.48, 3.10 and 3.58 predominantly contributed to the separation. In SHRSP, the urinary levels of taurine and creatine were found to be higher and the intensities of the unknown signals much lower than those in the Wistar Kyoto rats. Although the pathophysiological significance of these components remains to be elucidated, this study suggests that ^1H NMR-based metabonomics is a promising approach to provide new information on metabolic changes related to the pathophysiological processes of the genetically hypertensive rats. (*Hypertens Res* 2005; 28: 425–430)

Key Words: spontaneously hypertensive rat, metabonomics, nuclear magnetic resonance, urine

Introduction

Hypertension is a major risk factor for cardiovascular morbidity and mortality. In the majority of cases, the primary cause of hypertension remains unknown because of its complex nature as a polygenic, heterogenous and multifactorial disease, and the disorder is classified as essential hypertension (1, 2). Genetically hypertensive rat models provide a simple and accessible means of studying this complex disease (3). The spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP) are the most widely studied animal models of

hypertension, because some of their pathophysiological processes are similar to those of essential hypertension. The difference of genetic and metabolic features between these hypertensive rats and their normotensive controls, *i.e.*, Wistar Kyoto rats (WKY) is of great interest because it may provide insight into blood pressure regulation in human essential hypertension.

Although numerous studies have attempted to determine the genes responsible for the hypertension of SHR and SHRSP (4, 5), little progress has been made in identifying the specific genes. On the other hand, metabolic changes of SHR and SHRSP have not been fully studied due to a lack of suit-

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Table 1. Characteristics of the 12- and 26-Week-Old WKY and SHRSP

Age (week)	Strain	Body weight (g)	Blood pressure (mmHg)	
			Systolic blood pressure	Diastolic blood pressure
12	WKY (<i>n</i> =5)	319±17	132±10	93±5
	SHRSP (<i>n</i> =5)	250±7*	218±8*	148±13*
26	WKY (<i>n</i> =6)	432±27*	126±11	90±8
	SHRSP (<i>n</i> =5)	315±10 ^{†,§}	250±4 ^{†,§}	192±17 ^{†,§}

Values represent mean±SD. Statistical analysis was done using unpaired *t*-test. **p*<0.001 vs. 12-week-old WKY; [†]*p*<0.001 vs. 26-week-old WKY; [§]*p*<0.001 vs. 12-week-old SHRSP; [†]*p*<0.01 vs. 12-week-old SHRSP. WKY, Wistar-Kyoto rat; SHRSP, stroke-prone spontaneously hypertensive rat.

able techniques, despite the importance of such information in relating gene expression to protein functions and physiological events (6, 7). ¹H NMR spectroscopic analysis of biofluids allows for the simultaneous monitoring of a variety of low molecular weight endogenous metabolites from a range of intermediate metabolic pathways, without requiring pre-selection of measurable analytes (8), which contrasts markedly with most other techniques employed in biochemical analysis. Recently, a metabonomic approach using ¹H NMR and chemometric techniques has been developed (6, 9) to study metabolic differences associated with gene function and pathophysiological and toxicological stimuli. Although this combined technique has been successfully used to study several diseases and the toxicity of various drugs and other compounds (9), its application to understanding pathophysiological processes of hypertension has been limited (10). In this study, we have applied ¹H NMR-based metabonomic techniques to characterize the urinary metabolite profiles of SHRSP and WKY and to explore metabolite differences between these two strains.

Methods

Animals and Sample Collection

Six male SHRSP/Izm and WKY/Izm aged 10 weeks were obtained from the Disease Model Cooperative Research Association (Kyoto, Japan). Animal studies were conducted under approved guidelines and were reviewed by an institutional animal use committee. The animals were housed at 22°C with a 12-h light (7 AM–7 PM) and 12-h dark (7 PM–7 AM) cycle and free access to standard chow (CE-2; Clea Japan, Tokyo, Japan) and tap water. Rats were individually placed in metabolism cages (Natume, Tokyo, Japan) to collect urine from 3 PM for 24 h at 12 and 26 weeks of age. They were allowed free access to water but no food was given during urine collection. The vessels for urine collection were placed in Dewar vessels filled with dry ice, which permitted the collection of urine in the frozen state. Urine samples were filtered through DISMIC-25CS (hydrophilic, pore size 0.45 μm; Advantec Toyo, Osaka, Japan), and stored at –20°C prior to NMR spectroscopic analysis. Blood pressures were

measured using a tail-cuff method (BP-98A; Softron, Tokyo, Japan) immediately before urine collection.

NMR Spectroscopy

Deuterium oxide (100 μl) containing sodium 3-trimethylsilyl[2,2,3,3-²H₄]propionate (TSP, 215 μg) was added to a mixture of urine sample (350 μl) and 0.2 mol/l sodium phosphate buffer (pH 7.4, 350 μl). The mixture was filtered through DISMIC-13HP (hydrophilic, pore size 0.45 μm; Advantec Toyo) and then placed in a 5-mm o.d. NMR tube. ¹H NMR was measured on a Bruker DRX500 spectrometer (Bruker Biospin, Tsukuba, Japan) at a probe temperature of 300 K with the water resonance suppressed using a NOESYPRE-SAT pulse sequence (11). Sixty-four free-induction decays were collected into 65,536 data points with a spectral width of 10,000 Hz, an 11 μs pulse width (70° pulse angle), an acquisition time of 3.28 s, and a relaxation delay of 2 s to ensure full *T*₁ relaxation. Assignments of resonances were made on the basis of literature assignments and standard additions. Spectra were Fourier-transformed after application of a line-broadening function of 0.3 Hz and referenced to the chemical shift of TSP at δ 0.0.

Data-Reduction of NMR Data and Principal Components Analysis (PCA)

All NMR spectra were phased and baseline corrected. Data-reduction of NMR data and PCA were performed using AMIX software (version 3.0; Bruker Biospin). The NMR spectra (δ 0.5–9.5) were automatically reduced to 225 integrated segments of equal width (0.04 ppm). The region between δ 4.5 and δ 6.5 was removed prior to PCA to remove the effects of variation in the suppression of the water resonance and in the urea signal caused by partial cross solvent saturation *via* solvent exchanging protons. Following the removal of the regions, each spectral data set was normalized to the total sum of the integrals to compensate for variation in urine volumes and subjected to PCA. Scores plots of the principal components (PCs) were constructed to visualize separation of the urine samples between the groups, and the NMR spectral regions and endogenous metabolites that contribute

Fig. 1. Partial ^1H NMR spectra of urine samples from WKY (A) and SHRSP (B) at 12 weeks of age. Key: 1, succinate; 2 and 5, α -ketoglutarate (KG); 3, citrate (CA); 4, dimethylamine; 6 and 10, creatine (Cr); 7 and 11, creatinine (Cn); 8, trimethylamine-N-oxide (TMAO); 9, taurine (tau). U1, U2, U3, and U4 are unassigned resonances.

to the separation were identified from the values of the PC loadings, which indicate the importance of each variable to the separation.

Statistical Analysis

Data are expressed as the means $\pm$ SD. Statistical analysis was done using an unpaired *t*-test. Differences with *p*-values of <0.05 were considered statistically significant.

Results

The body weights and blood pressures are summarized in Table 1. The body weights of the 12- and 26-week-old SHRSP were 22% and 27% lower, respectively, than those of the age-matched WKY. The mean values of systolic blood pressure rose more than 200 mmHg in the 12- and 26-week-old SHRSP and much higher than in any WKY group.

Typical ^1H NMR spectra of whole urine samples from the hypertensive and normotensive rats are shown in Fig. 1. These highly complex spectra include many signals from a wide range of chemical classes of endogenous metabolites, and provide information on the relative activities of many cellular biochemical pathways. The profiles of the NMR spectra had many similar characteristic peaks, and visual comparison of the spectra was difficult because of inter-animal variations in the urine composition and the complexity of the spectra, coupled with differences in the volume of urine excreted. Thus, PCA was employed to determine whether it was possi-

ble to distinguish between samples of different groups based on their NMR spectra, and hence define a relationship between the urinary profile and blood pressure. The scores plot of PC1 vs. PC2 showed that the urinary profiles of samples obtained from SHRSP were clearly different from the profiles obtained from WKY at both ages, as shown in Fig. 2. The samples from the hypertensive and normotensive strains were separated along the first PC, with the former having higher scores. In addition, the scores plot showed that the samples were separated into two distinct clusters relating to the age in each strain on the PC2 axis, with the higher-aged group having lower scores, except in the case of one sample obtained from SHRSP at 26 weeks of age. Samples obtained from WKY formed a tighter cluster than did those from SHRSP.

In order to determine the spectral regions and components contributing to the separation on the PCA maps, the PC loadings were examined. As shown in Fig. 3, the NMR spectral regions (δ 3.22–3.26, 3.26–3.30, 3.34–3.38, 3.38–3.42, 3.42–3.46, and 3.90–3.94) in the loadings plot were in positions similar to those of the samples from the hypertensive strain in the scores plot, *i.e.*, to the right-hand side in either plot. Conversely, the NMR spectral regions (δ 2.42–2.46, 2.46–2.50, 3.06–3.10, 3.10–3.14, and 3.54–3.58) were mapped to the opposite side of the loadings plot as compared to the mapping of the samples from the hypertensive strain in the scores plot, *i.e.*, to the left-hand side of the plot. The NMR spectral regions, δ 3.22–3.26, 3.26–3.30, 3.38–3.42, 3.42–3.46, and 3.90–3.94, corresponded to taurine and creatine, whereas the

Fig. 2. PC scores plot derived from the ^1H NMR spectra of urine samples obtained from WKY and SHRSP at 12 and 26 weeks of age. Open circle, 12-week-old WKY; open lozenge, 26-week-old WKY; closed circle, 12-week-old SHRSP; closed lozenge, 26-week-old SHRSP.

spectral regions δ 2.42–2.46, 2.46–2.50, 3.06–3.10, 3.10–3.14, 3.34–3.38, and 3.54–3.58 included as yet unidentified signals (U1–U4), as shown in Fig. 1. Thus, the separation of the hypertensive strain from the normotensive strain was shown to be mainly attributable to the relatively high urinary levels of the taurine, creatine and U3 signals, and the remarkably low urinary levels of the three unknown signals (U1, U2, U4) in the former strain. The formation of clusters relating to aging (Fig. 2) was found to be mainly due to the high levels of creatinine in the higher-age groups, since the loadings corresponding to creatinine were negatively correlated with the PC2 scores. These alterations were confirmed by visual inspection of the NMR spectra.

The molar ratios of creatine and taurine to creatinine were determined by comparison of the signal areas, taking into account the molar equivalent number of protons contributing to the measured signals: creatine, δ 3.93 (singlet, CH_2); taurine, δ 3.43 (triplet, CH_2); and creatinine, δ 4.06 (singlet, CH_2). As shown in Table 2, the creatine levels of SHRSP were significantly higher than those of WKY at both ages. The taurine levels of SHRSP were higher than those of WKY. SHRSP at 12 weeks of age had significantly elevated levels of taurine, although those of SHRSP at 26 weeks of age had a relatively large individual variation and did not attain statistical significance.

Discussion

Urine is a complex biofluid containing hundreds of components, many of which have not been chemically identified. Hence, current analyses target only recognized measurable substances, and any potential information that may be

Fig. 3. Loadings plot of the PCA model shown in Fig. 2. Each circle represents a spectral region covering 0.04 ppm, and the middle chemical shift of the region and the signal corresponding to the region are given beside each circle. Positive loadings indicate relatively higher integral values for that spectral region in the SHRSP samples compared with the WKY samples, whereas negative coefficients indicate lower integral values. The magnitude of the loadings represents the relative importance of each spectral region on the separation achieved in Fig. 2. Key: see Fig. 1.

obtained from the remainder is lost. The use of metabonomic procedures, which utilize NMR spectroscopy combined with PCA, is unique in its ability to detect and characterize numerous metabolic components even when their chemical identities are initially unknown. An additional advantage of the procedures is that the results obtained are not limited by preconceptions, and new information about the nature and causes of the pathophysiological changes is expected to be obtained.

PCA of the ^1H NMR spectral data from WKY and SHRSP indicated excellent separation between the two strains in terms of metabolic profiles, as demonstrated by the clustering observed in the PC scores plot (Fig. 2). This observation implies that the chemical constituents of the urine samples from the two strains were significantly different. The loadings plot indicated that some urinary metabolites contributed to the separation in the PCA map. These results may suggest the existence of a relationship between blood pressure and the urinary metabolic profile.

Taurine had the greatest variation between the hypertensive and normotensive strains, with its urinary level being higher in the former strain. Taurine is present in high concentrations in mammalian tissues and is known to exert beneficial effects. For example, the substance has been reported to attenuate elevations in the blood pressure of hypertensive patients as well as SHR and SHRSP, although the exact mechanisms are not totally understood (12). There have been conflicting reports in the literature concerning the differences in plasma taurine

Table 2. Urinary Excretion of Taurine and Creatine

Group	Molar ratios to creatinine	
	Taurine	Creatine
12 weeks of age		
WKY (n=5)	0.34±0.09	0.08±0.03
SHRSP (n=5)	0.57±0.13*	0.24±0.12*
26 weeks of age		
WKY (n=6)	0.28±0.04	0.05±0.01
SHRSP (n=5)	0.70±0.35	0.26±0.15**

Molar ratios of urinary taurine and creatine to creatinine were calculated based on the integral intensities of the NMR signals observed in urine samples collected for 24 h. Values are mean±SD. Statistical analysis was done using unpaired *t*-test. **p*<0.05 vs. 12-week-old WKY; ***p*<0.05 vs. 26-week-old WKY. WKY, Wistar-Kyoto rat; SHRSP, stroke-prone spontaneously hypertensive rat.

levels between WKY and SHR or SHRSP (13, 14). To the best of our knowledge, however, there has been no report comparing the urinary level of taurine between WKY and SHR or SHRSP. Kohashi and Katori (15) have reported that the urinary excretion of taurine was decreased in patients with essential hypertension, which is contrary to our results in SHRSP. However, it is generally difficult to compare urinary levels of taurine in humans because of a remarkable individual variation that is based on various factors (16). In particular, humans are more dependent on dietary sources of taurine than rats, since humans are relatively poor synthesizers of taurine (17). Therefore, further investigations may be required to clarify the changes in urinary taurine in essential hypertension.

Creatine levels were found to be higher in SHRSP at 12 and 26 weeks of age than in age-matched WKY. Creatine is formed by the transfer of a methyl group of S-adenosylmethionine to guanidinoacetic acid, in the course of hepatic synthesis of cysteine from methionine. Thus excess creatine may be formed when the rate of cysteine synthesis is high because of the numerous defensive processes requiring cysteine. A similar mechanism has been proposed for hepatotoxin-induced creatinuria in rats (18, 19). Thus it is possible that the creatinuria observed in SHRSP resulted from the accelerated biosynthesis of cysteine, although in general creatinuria is caused simply by leakage of creatine from damaged muscle. Moreover, the taurinuria observed in SHRSP might also be explained by the accelerated cysteine synthesis, since taurine formation may be enhanced by the increased availability of substrate cysteine in the liver. As shown in Fig. 4, the urinary levels of creatine and taurine in SHRSP were highly correlated. These findings may suggest that the creatinuria and taurinuria occurred in SHRSP at the same time based on the accelerated cysteine synthesis, although it is difficult to exclude the possibility of altered renal handling of creatine and taurine.

Fig. 4. The linear relationship between taurine and creatine excretion in the urine samples collected from SHRSP. Taurine and creatine excretion in SHRSP are expressed relative to creatinine excretion by means of molar ratios. The correlation coefficient $r=0.60$. The data from 12- and 26-week-old WKY are also shown as a reference. Open circle, 12-week-old WKY; open lozenge, 26-week-old WKY; closed circle, 12-week-old SHRSP; closed lozenge, 26-week-old SHRSP.

In summary, using ^1H NMR-based metabonomic approaches, urine samples of SHRSP with established hypertension were separated from those of WKY according to their metabolic profiles, and some characteristic metabolic features of SHRSP were revealed, *i.e.*, increases in taurine and creatine and decreases in some unknown metabolites resonating at around δ 2.48, 3.10, and 3.58. This study seems to indicate that metabonomics of SHRSP is a promising approach to provide new information on metabolic changes related to hypertension. Although the pathophysiological significance of these changes remains to be elucidated, further analysis of the molecular basis of the spectral differences, including identification of the unknown metabolites, would provide insight into the mechanistic processes involved in the pathogenesis of hypertension and its complications.

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