Effects of two formulations for overcoming oketsu on vascular function and expression patterns of plasma proteins in spontaneously diabetic rats

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We investigated the effects of keishibukuryogan and tokishakuyakusan, which are representative formulations for overcoming oketsu, on vascular function and expression patterns of plasma proteins in spontaneously diabetic rats. Twenty-one- to 24-week-old male WBN/Kob rats were maintained for 18 weeks on a diabetes-accelerated feed, and received standard (diabetes-accelerating) chow containing 3% (wt/wt) keishibukuryogan or tokishakuyakusan for 25 weeks. There was no significant change in body weight or blood glucose among the groups. Acetylcholine-induced endothelium-dependent relaxation of the keishibukuryogan group significantly increased compared to that of controls. Xanthine/xanthine oxidase-induced contraction of the tokishakuyakusan group and phospholipase A_2-induced contraction of the keishibukuryogan and tokishakuyakusan groups significantly decreased compared to the controls. Transit time of whole blood tended to decrease in the tokishakuyakusan group compared to controls. NO_2^-/NO_3^- in the keishibukuryogan and tokishakuyakusan groups significantly decreased compared to controls. A study using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) demonstrated that five and eight peaks had significantly changed peak intensities in plasma of rats treated with keishibukuryogan and tokishakuyakusan, respectively, as compared to the controls. Thus, two representative formulations for overcoming oketsu with different mechanisms of action had favorable effects against vascular dysfunction. Altered plasma protein levels were commonly observed in the rats administered these two formulations. Our study using ProteinChip technology may be useful for the evaluation of the relationship between the efficacy and the profiling of plasma protein expression after administration Kampo medicines, thus leading to the understanding of "Sho" in Kampo medicine.

Key words keishibukuryogan, tokishakuyakusan, vascular function, WBN/Kob rat, surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), protein chip.

Introduction

WBN/Kob rats are spontaneously diabetic rats that live for a long time with hyperglycemia. Several hyperglycemia-induced complications have been observed in WBN/Kob rats,[1)] including vascular dysfunction and diabetic nephropathy. This condition is called "oketsu," and is defined as a state of insufficient blood circulation and blood stasis resulting in diabetic retinopathy, etc. We have reported[2)] that this vascular dysfunction was improved by the Kampo medicine, keishibukuryogan. Tokishakuyakusan is also an important formulation for overcoming oketsu and has been reported to improve blood circulation.[3)] Clinically, keishibukuryogan and tokishakuyakusan are based on the oriental concept of Yin-yang and hypofunction-hyperfunction. However, the effects these formulations have on host function are not clear in terms of modern medicine. Furthermore, the differences in host responses are thought to affect variations in the "Sho" diagnosis in Kampo medicine.

In previous reports, we investigated the expression of genes and proteins associated with various diseases in order to clarify the scientific basis of "Sho" in Kampo medicine. Administration of the Kampo medicine hachimijiogan, which has been mainly used for fatigue, exhaustion, nephritis, and diabetes mellitus, was effective at reducing the expression of diabetic nephropathy but not at reducing blood glucose levels.[10)] The expression patterns of plasma proteins using ProteinChip array revealed that several proteins in the plasma may be involved in the development and/or progression of diabetic nephropathy in WBN/Kob rats and the efficacy of hachimijiogan.[10)]

In the present study, we investigated the effects of keishibukuryogan and tokishakuyakusan on vascular function in spontaneously diabetic rats. We also examined the expression patterns of plasma proteins in these rats using

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ProteinChip technology to identify multiple biomarkers associated with "Sho" diagnosis.

Materials and Methods

Animals. Twenty-one to 24-week-old male WBN/Kob rats obtained from Sankyo Labo Service (Toyama, Japan) were used. They were kept in an animal room at an ambient temperature of 23 ± 1 ℃ under a 12-h light-dark cycle. They were maintained for 18 weeks on a feed that accelerated diabetes (Labo MR-DBT, Nosan Corporation, Yokohama, Japan). Their diabetes was confirmed when their fasting blood sugar reached above 200 mg/dl.

Experimental protocols met the "Guidelines for Animal Experimentation" approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society.

Drugs. Powdered keishibukuryogan and tokishakuyakusan were purchased from Uchida Wakanyaku (Tokyo, Japan). Keishibukuryogan consisted of equal amounts of the following five crude drugs: 5g of Cinnamoni Cortex (*Cinnamomum cassia* BLUME), 5g of Hoelen (*Poria cocos* WOLF), 5g of Moutan Cortex (*Paeonia suffruticosa* ANDREWS), 5g of Persicae Semen (*Purnus persicae* BATASCH), and 5g of Paeonia Radix (*Paeonia lactiflora* PALL) in 25 g of keishibukuryogan. Tokishakuyakusan consisted of the following six crude drugs: 6 g of Alismatis Rhizoma (*Alisma orientale* JUZEPCZUK), 5 g of Paeoniae Radix (*Paeonia lactiflora* PALL), 4 g of Atractylodis Rhizoma (*Atractylodes lancea* DE CANDOLLE), 4 g of Hoelen (*Poria cocos* WOLF), 3 g of Cnidii Rhizoma (*Cnidium officinale* MAKINO), and 3 g of Angelicae Radix (*Angelica sinensis* DIELS) in 25 g of tokishakuyakusan.

Drug treatment. WBN/Kob rats were randomly assigned to three groups (control, 3% keishibukuryogan, 3% tokishakuyakusan). Rats in the control group received standard (diabetes-accelerating) chow for 25 weeks. Rats in the 3% keishibukuryogan and 3% tokishakuyakusan groups received standard chow containing 3% (wt/wt) keishibukuryogan and tokishakuyakusan for 25 weeks, respectively. In terms of total chow volume, the drug doses used in this study corresponded to about 10 times the clinical doses, respectively.

Body weight and blood sugar measurement. Body weight and blood glucose were measured at two-week intervals from the baseline period until sacrifice. Blood glucose was determined using commercial reagents (Glucose CII-Test Wako obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan).

Relaxation experiments. The rats were anesthetized (50 mg/kg i.p. pentobarbiturate) and sacrificed by drawing blood from the heart. A section of the thoracic aorta was carefully cleaned of fat and connective tissues, and 3-mm ring preparations were made. The rings were mounted on steel hooks in a Magnus chamber (UC-5TD, Kishimoto, Kyoto, Japan). One end of the aorta was attached to a force-displacement transducer (UM-203, Kishimoto) so that its isometric contraction could be recorded (T-634, Niko Bioscience, Tokyo, Japan). Baths were filled with 5 ml of Krebs solution of the following composition (mM): NaCl 120, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5 and glucose 10.0. The solution was maintained at 37℃ and bubbled continuously with 5% CO_2 in O_2 at pH 7.4. The rings were equilibrated for 40 min at an initial resting tension of 1 g. During this time, the Krebs solution in the tissue bath was replaced every 15 min. The rings were then precontracted with 5×10^{-7} M noradrenaline (NA). For endothelium-dependent relaxations, vessels were relaxed with acetylcholine (Ach) (10^{-9} to 10^{-4} M). To study the endothelium-independent relaxation of vascular smooth muscle, vessels were relaxed with sodium nitroprusside (SNP) (10^{-9} to 10^{-4} M). Relaxation was expressed as a percentage of the decrease in maximal tension obtained by NA-induced contraction.

Contraction experiments. *Contraction induced by xanthine/xanthine oxidase.* To determine the endothelium-dependent contraction of the aorta induced by oxygen-derived free radicals, we placed a segment of an aorta in medium containing xanthine (10^{-4} M) and 10^{-4} M N^G-nitro-L-arginine methylester (L-NAME). Oxygen-derived free radical-induced endothelium-dependent contraction of aortas was determined by the addition of 10 mU/ml xanthine oxidase to medium containing xanthine. This contraction was expressed as a percentage of the relative increase to the maximal tension obtained by 60 mM KCl-induced contraction.

Contraction induced by phospholipase A_2 (PLA_2). To examine the effect against thromboxane A_2 - and prostaglandin H_2-induced contraction, PLA_2 (1 U/ml) was administered and transient contraction was induced in medium containing 10^{-4} M L-NAME. The contraction was expressed as a percentage of 60 mM KCl maximum contraction.

Contraction induced by angiotensin II (Ang II). To examine the effect against Ang II-induced contraction, Ang II (10^{-8} to 10^{-6} M) was administered and transient contraction was induced in medium containing 10^{-4} M L-NAME. The contraction was expressed as a percentage of 60 mM KCl maximum contraction.

Measurement of plasma triglyceride, lipid peroxides, fibrinogen and nitric oxide (NO). Triglyceride was determined by the standard method. Lipid peroxides were measured by the Yagi method.[4] Plasma fibrinogen was measured by the thrombin time method.[5] NO is an extremely unstable molecule and rapidly undergoes oxidative degradation to the stable inorganic nitrogen oxides NO_2^-/NO_3^-, which were used here as indices of in vivo NO generation. Serum NO_2^-/NO_3^- was measured with an automated system (ENO-10; EICOM Co., Kyoto, Japan) based on the Griess reaction method.

Microchannel array flow analysis. The transit time of whole blood through the microchannel array was measured and used as a marker of blood fluidity. The detailed procedures and apparatus of the microchannel analysis (Microchannel Array Flow Analyzer [MC-FAN], type KH-2, Hitachi Haramachi Electronics Co. Ltd., Hitachi, Tokyo, Japan) have been described previously.[6] Briefly,

microgrooves formed in the surface of a single crystal silicon substrate were converted to leak-proof microchannels by covering them tightly with an optical flat glass plate. The microgrooves in the silicon microchannel chip resembling the size of capillaries (Bloody-6-5, 8736 channels; width, 5 μm; depth, 4.5 μm; length, 20 μm - Hitachi Haramachi Electronics Co. Ltd., Hitachi, Tokyo) were prefilled with saline. Heparinized whole blood samples were forced to flow through the microchannels under a pressure difference of 20 cm H_2O. To assess the filterability of whole blood, the transit time for 100 μl of blood was determined. These measurements were performed immediately after blood sampling at room temperature between 20℃ and 25℃. MC-FAN was calibrated with saline before each new measurement. Blood passage through an individual channel was observed and recorded using a video microscope system (WAT-231S, WATEC, Tokyo, Japan).

Plasma sample preparation for SELDI protein profiling. Plasma samples were centrifuged at 3000 rpm for 10 min to remove insoluble debris and stored at -80℃ until used in the SELDI profiling study. Samples were thawed and then diluted (1:10 v/v in 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholyte).

SELDI protein profiling. Various chip properties (hydrophobic, anionic, cationic, and metal binding) were initially evaluated to determine which affinity chemistry provided the best serum profiles in terms of number and resolution of proteins. The cationic CM10 Chip Array (Ciphergen Biosystems, Fremont, CA) produced the best results. Protein Chip Arrays were assembled into a deep-well type bioprocessor assembly (Ciphergen Biosystems) equipped in a Laboratory Automation Workstation Biomek® 2000 (Beckman Coulter, Fullerton, CA). Prior to sample loading, CM10 Arrays were equilibrated with 150 μl of buffer (100 mM sodium acetate, pH 5.0) into each well and then prewashed two times for 5 min on a shaker at room temperature. To the arrays, 90 μl of buffer (100 mM sodium acetate, pH 5.0) and 10 μl of diluted sample were added into each well, and then the arrays were incubated for 30 min on a shaker at room temperature, and washed three times with 150 μl of buffer for 5 min on a shaker at room temperature. After rinsing two times with 200 μl of deionized water, the arrays were removed from the bioprocessor assembly and air-dried. One microliter of 50% solution of the energy-adsorbing molecule (EAM): alpha-cyano-4-hydroxy cinnamic acid (CHCA) (Ciphergen Biosystems) in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid was applied two times onto each ProteinChip Array, letting the array surface air-dry between each CHCA application.

The protein chip arrays were analyzed using a ProteinChip Biology System Reader (Model PBS-IIc; Ciphergen Biosystems). Spectra were collected at a laser intensity of 165 and a detector sensitivity of 6 in the positive ion mode. The protein masses were calibrated externally using purified peptide and protein standards (Ciphergen Biosystems). A mass range from 2000-10000 Da was selected for analysis because this range contained the majority of the resolved protein/peptides. Molecular masses from 0-2000 Da were eliminated from analysis because this area contains adducts of EAM and possibly other chemical contaminants.

Data analysis. Data on vascular function and blood samples are presented as mean ± standard error (S.E.). Statistical comparisons were performed using Fisher's PLSD test and repeated measures ANOVA. The level of statistical significance was defined as $p<0.05$. Spectra were analyzed with ProteinChip Software (Version 3.2.0; Ciphergen Biosystems). In order to use the intensities as indicators of the relative abundance of peptide in the sample, baselines had to be subtracted and the intensities normalized. Normalization was performed by total ion current normalization function following the software instructions. Biomarker Wizard (Ciphergen Biosystems) was then used to identify corresponding peaks in each spectrum within 0.3% of the mass. The signal-to-noise ratio was set to 2 for the first pass and to 2 for the second pass. We used the Mann-Whitney *U* test for nonparametric data sets to compare the peak intensities of the protein profiling results from the different groups.

Results

Effect of keishibukuryogan and tokishakuyakusan on vascular functions in spontaneously diabetic rats. There were no discernible differences in the body weight or blood glucose among the control, keishibukuryogan and tokishakuyakusan groups (Table 1). As shown in Figure 1A, Ach-induced endothelium-dependent relaxation, reaching 10^{-6} M, and relaxation of the keishibukuryogan group were significantly increased as compared to the control

Table 1 Characteristics of WBN/Kob rats in different experimental groups.

Group		Control	Keishibukuryogan	Tokishakuyakusan
Body weight	(g)	360 ± 11.4	333 ± 11.2	332 ± 7.2
Blood sugar	(mg/dl)	736 ± 32.0	590 ± 74.5	655 ± 44.0
Triglyceride	(mg/dl)	202 ± 38.1	163 ± 25.9	155 ± 30.4
Lipid peroxide	($\times 10^{-6}$M)	5.0 ± 2.8	6.1 ± 1.6	6.2 ± 2.8
Fibrinogen	(mg/dl)	287 ± 34.3	278 ± 29.6	265 ± 21.8
Serum No_2^-/No_3^-	($\times 10^{-5}$M)	20.3 ± 2.21	9.8 ± 1.15[a]	7.7 ± 0.81[a]
Microchannel transit time of whole blood	(sec)	69.0 ± 1.0	65.6 ± 2.0	64.5 ± 1.9[b]

Each value is mean ± S.E. of 7 rats. [a]$p<0.05$, [b]$p<0.1$ vs WBN/Kob control group.

group ($p<0.05$). Maximum relaxations were 45.9 ± 1.1%, 59.7 ± 1.3% and 52.0 ± 1.2 % in control, keishibukuryogan and tokishakuyakusan groups, respectively (mean ± S.E., n=7). In endothelium-independent relaxation, there was no significant difference in SNP among the three groups (Fig. 1B).

Xanthine oxidase-induced contraction of the tokishakuyakusan group was significantly decreased as compared to the control group ($p<0.05$). The contractions at 10 mU/ml xanthine oxidase were 37.3 ± 3.2%, 28.6 ± 3.5% and 25.4 ± 3.8% in the control, keishibukuryogan and tokishakuyakusan groups, respectively (Fig. 2). PLA_2-induced contraction in both the keishibukuryogan and tokishakuyakusan groups was significantly decreased as compared to the control group ($p<0.05$). Contractions at 1 U/ml PLA_2 were 39.0 ± 3.8%, 27.5 ± 2.4% and 27.7 ± 1.4% in the control, keishibukuryogan and tokishakuyakusan groups, respectively (Fig. 2). Ang II-induced contraction in both the keishibukuryogan and tokishakuyakusan groups was significantly decreased as compared to the control group ($p<0.05$). Contractions at 10^{-6} M Ang II were 28.6 ± 2.6 %, 20.8 ± 2.8% and 20.1 ± 2.3% in the control, keishibukuryogan and tokishakuyakusan groups, respectively (Fig. 3).

Triglyceride, lipid peroxide and fibrinogen showed no significant differences among the control, keishibukuryogan and tokishakuyakusan groups. NO_2^-/NO_3^- decreased significantly in the keishibukuryogan and tokishakuyakusan groups compared to the control group. The effects of keishibukuryogan and tokishakuyakusan on blood fluidity were evaluated by the microchannel transit time of whole blood, measured by MC-FAN. Transit time of whole blood tended to decrease in the tokishakuyakusan group compared

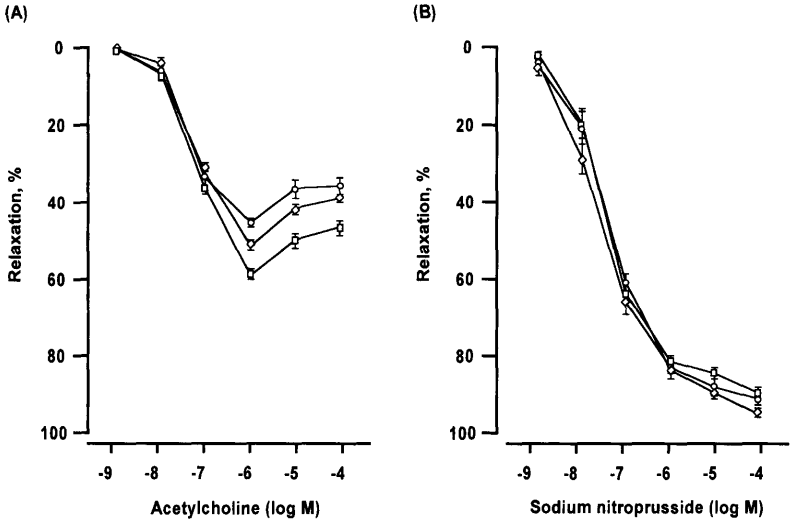

Figure 1 Graph showing A) endothelium-dependent relaxation in response to acetylcholine, B) endothelium-independent relaxation in response to sodium nitroprusside in the aorta of WBN/Kob rats treated for 25 weeks. WBN/Kob control group (○), keishibukuryogan group (□), and tokishakuyakusan group (◇). Values are expressed as a percentage of the decrease in maximal tension contracted with 5×10^{-7} M NA. Shown is the mean ± S.E. of seven determinations. Differences between WBN/Kob control and keishibukuryogan in graph (A) were statistically significant ($p<0.05$, n=7).

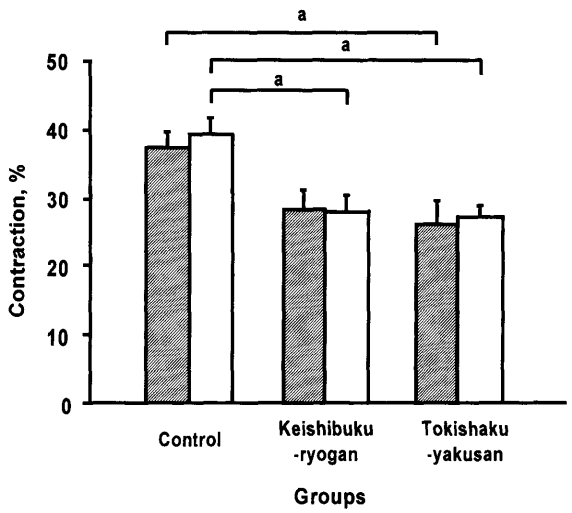

Figure 2 Vasocontraction treatment on xanthine oxidase (10 mU/ml) in the presence of xanthine (10^{-4}M) (■) and phospholipase (1 U/ml) (□) in the aorta of WBN/Kob rats treated for 25 weeks. All aortas had intact endothelium and had been treated with L-NAME. Contraction was expressed as a percentage of 60 mM KCl maximum contraction. Asterisks indicate significant differences from WBN/Kob control group ($^a p<0.05$, mean ± S.E., n=7).

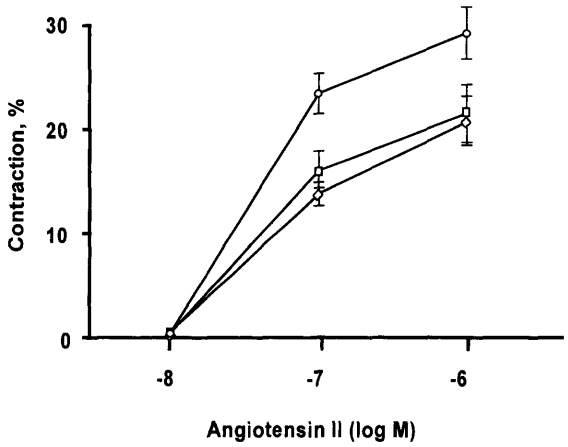

Figure 3 Graph showing angiotensin II induced-contraction in aorta of WBN/Kob rats treated for 25 weeks. WBN/Kob control group (○), keishibukuryogan group (□), and tokishakuyakusan group (◇). Contraction was expressed as the percentage of 60 mM KCl maximum contraction. Shown is the mean ± S.E. of seven determinations. Differences between WBN/Kob control and keishibukuryogan and tokishakuyakusan are statistically significant ($p<0.05$, n=7).

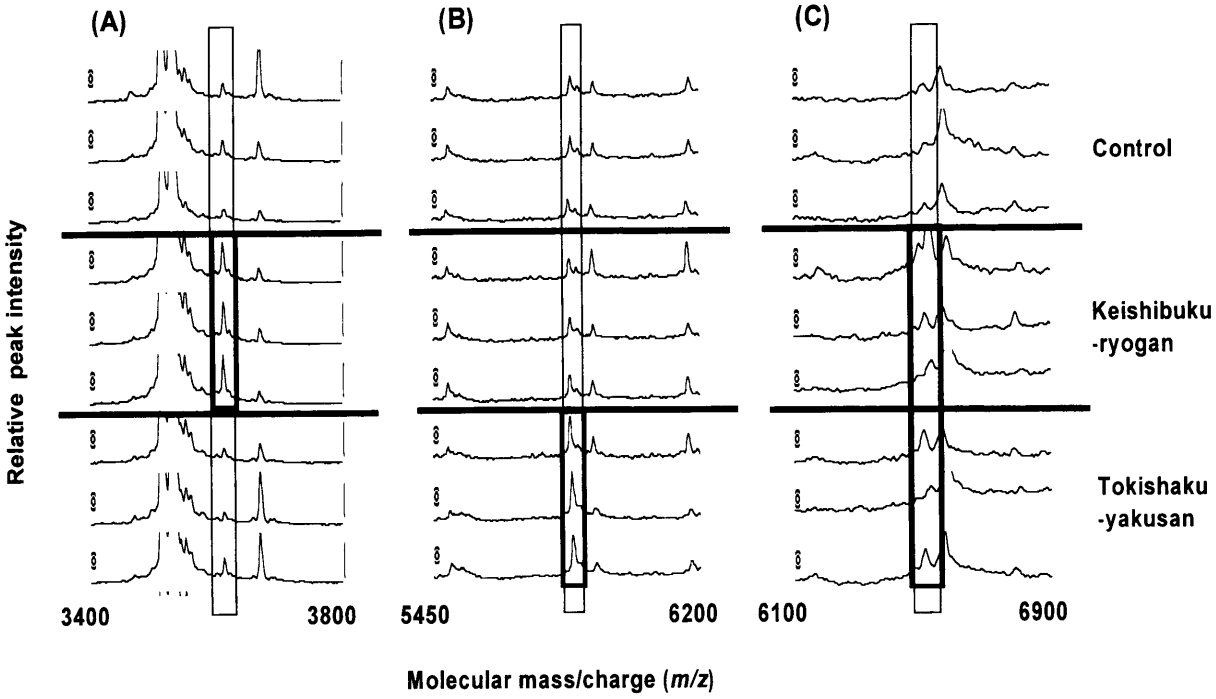

Figure 4 Typical peaks recognized by administration of keishibukuryogan and tokishakuyakusan. A) A typical peak elevated by administration of keishibukuryogan. B) A typical peak elevated by administration of tokishakuyakusan. C) A typical peak elevated by administration of both keishibukuryogan and tokishakuyakusan.

Table 2 Summary of *m/z* values and peak intensities changed significantly in the diabetic rats by keishiburyogan and tokishakuyakusan

	Peak Intensity (mean ± S.D.)		
m/z	Control	Keishibukuryogan	Tokishakuyakusan
3264.772	0.208 ± 0.102	0.309 ± 0.213	0.463 ± 0.400
3280.847	1.296 ± 0.187	1.138 ± 0.210	1.274 ± 0.372
3612.813	8.356 ± 2.445	11.998 ± 4.389	8.844 ± 3.864
3899.419	0.957 ± 0.146	1.022 ± 0.335	1.256 ± 0.183
4433.000	1.229 ± 0.302	1.506 ± 0.459	1.722 ± 0.623
4459.540	0.364 ± 0.142	0.519 ± 0.128	0.524 ± 0.185
4490.796	0.114 ± 0.093	0.221 ± 0.096	0.193 ± 0.104
5837.196	3.937 ± 0.696	4.124 ± 0.887	5.006 ± 0.690
5854.691	1.642 ± 0.202	1.742 ± 0.249	1.835 ± 0.203
6506.587	1.149 ± 0.313	1.385 ± 0.280	1.618 ± 0.665

Underline : $p<0.05$ vs. control by Mann-Whitney *U* test.

to the control group (Table 1).

Changes in plasma protein profiling of diabetic rats after oral administration of keishibukuryogan and tokishakuyakusan. We investigated the influence of keishibukuryogan and tokishakuyakusan on the expression patterns of plasma proteins in WBN/Kob rats at 67 weeks of age. Spectral analysis of samples (control group, n=7; keishibukuryogan group, n=7; tokishakuyakusan group, n=7) was performed in duplicate using the ProteinChip software program. Approximately 230 peaks per spectrum were detected in the 2000-10000 Da mass range. Representative spectra of plasma proteins of WBN/Kob rats are shown in Fig. 4. As summarized in Table 2, administration of keishibukuryogan led to significant changes in the intensities of the five peaks (*m/z* 3280, 3612, 4459, 4490 and 6506), and that of tokishakuyakusan led to significant elevation in the intensities of eight peaks (m/z 3264, 3899, 4433, 4459, 4490, 5837, 5854, and 6506), compared with control diabetic rats. Three of the peaks (m/z 4459, 4490 and 6506) were elevated by the administration of both keishibukuryogan and tokishakuyakusan.

Discussion

The WBN/Kob rat is a spontaneously insulin-dependent diabetic rat, in which the diabetes is caused by pancreatitis. This animal has a long life span, so several hyperglycemia-induced complications have been observed.[1] Several vascular dysfunctions have also been observed in WBN/Kob rats, such as endothelial dysfunction.[7] In this regard, some Kampo formulations for overcoming oketsu have been used to treat gynecological, psychiatric, and dermatological diseases *etc.* For arteriosclerosis, vascular protective effects that improve blood circulation have been reported for formulations used for overcoming oketsu.[8,9] Recently, we demonstrated that keishibukuryogan improved vascular dysfunction in WBN/Kob rats through improvement of endothelial function, suppression of vasocontraction, and decreasing blood viscosity.[2]

In the present study, we examined the effects of tokishakuyakusan in WBN/Kob rats in comparison with the effects of keishibukuryogan. The results were that keishibukuryogan was effective at improving endothelial function more than tokishakuyakusan, while tokishakuyaku-

san was effective at improving blood fluidity more than keishibukuryogan. Because the different Kampo formulations caused different responses in vascular function even in the same model, it is thought that the responses of the body also vary according to the various kinds of Kampo formulations. This variety in body responses is explained by the Kampo diagnosis of "Sho".

Since Kampo formulations are generally prepared from the combination of several crude drugs, these drugs are believed to have harmonization effects, which results in different effects than each individual crude drug. Therefore, in order to evaluate the influence of Kampo formulations on various diseases that result from multiple factors, an inclusive analytical method, such as ProteinChip technology, may be useful for profiling biological mixtures and identifying multiple biomarkers associated with diseases. We recently reported that hachimijiogan prevented renal dysfunction-induced hyperglycemia in WBN/Kob rat, and that several plasma proteins may be involved in the progression of disease and the efficacy of hachimijiogan.[10] Here we identify one of these proteins and evaluate the relationship between the efficacy of hachimijiogan and expression profiling.

The expression pattern of plasma proteins by SELDI profiling revealed that five peaks in the keishibukuryogan-treated group and eight peaks in the tokishakuyakusan-treated group were significantly changed in the 2000-10000 Da mass range compared to the control group (Table 2). Three peaks at *m/z* 4459, 4490 and 6506 were observed to be commonly elevated after the oral administration of keishibukuryogan and tokishakuyakusan. On vascular functions and blood fluidity particular effects were seen with keishibukuryogan and tokishakuyakusan. Further studies will be needed to examine the correlation between these effects and expression patterns of plasma proteins in detail. In Kampo medicine, keishibukuryogan is used for the patient who is Yang and hyperfunctional, while tokishakuyakusan is used for the patient who is Yin and hypofunctional. Thus, the differences of "Sho" for both medicines may be related to the differences in expression profiling of proteins in plasma as well as the mechanism of action.

Table 3 shows the summary of our present and previous studies on the expression patterns of plasma proteins in spontaneously diabetic rats after oral administration of three Kampo medicines using SELDI-TOF-MS. Administration of hachimijiogan significantly decreased the increased levels of six plasma proteins as compared with the control group.[10] These six plasma proteins associated with hachimijiogan were evidently different at the *m/z* level from those of formulations for overcoming oketsu, keishibukuryogan and tokishakuyakusan (Table 3). As increase in the intensities of three peaks at *m/z* 4459, 4490 and 6506 was commonly observed in both keishibukuryogan and tokishakuyakusan groups, the expression of these proteins may be specific or characteristic in the formulations for overcoming oketsu. These results may provide an important basis for clarifying "Sho" and the determination of "Sho"-directed formulations. On the other hand, shakuyaku and bukuryo are commonly contained in keishibukuryogan and tokishakuyakusan; keishi, botanpi and bukuryo in keishibukuryogan and hachimijiogan; and takusha and bukuryo in tokishakuyakusan and hachimijiogan. Thus, further study will be needed to examine whether the combination of crude drugs contained in the three formulations can be associated with the changes in expression patterns of plasma proteins after administration of these formulations.

Table 3 Changes in expression of plasma proteins of spontaneously diabetic WBN/Kob rats administered keishiburyogan, tokishakuyakusan and hachimijiogan

Group / *m/z*	Keishibukuryogan	Tokishakuyakusan	Hachimijiogan
3264		↑	
3280	↓		
3612	↑		
3899		↑	
4433		↑	
4459	↑	↑	
4490	↑	↑	
4679			↓
4733			↓
4808			↓
5837		↑	
5854		↑	
6506	↑	↑	
9058			↓
9323			↓
9465			↓

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Japanese abstract

自然発症糖尿病モデルであるWBN/Kobラットに代表的な駆瘀血薬である桂枝茯苓丸と当帰芍薬散を長期間投与し，血管機能とタンパク発現に及ぼす影響を検討した。方法は，WBN/Kobラット（雄，24週令）を18週間飼育し糖尿病発症を確認した後，対照群，3％桂枝茯苓丸（KB）群，3％当帰芍薬散（TS）群の3群に分け，さらに25週間飼育した。飼育後，胸部大動脈を摘出しOrgan bath法を用いacetylcholine（Ach）による血管弛緩作用，xanthine/xanthine oxidase（X/XOD）投与による血管収縮作用等を検討した。同時に，血液流動性，血漿脂質，NO代謝物等の測定とSELDI-TOF-MSによる血漿プロテオーム解析を施行した。結果は，対照群とKB，TS群の3群間において，体重と血糖値に有意な差を認めなかった。Achによる内皮依存性血管弛緩率はKB群で対照群に対し有意に弛緩率の増加を認めた。X/XOD投与による血管収縮率はTS群で，PLA_2投与による血管収縮率はTS，KB群の両群で対照群に対し収縮率の減少を認めた。血液流動性はTS群で対照群に対し改善傾向を認め，NO代謝物はKB，TS群の両群で対照群に対し有意に減少した。血漿プロテオーム解析により，対照群に比較しKB群では5個，TS群で8個のタンパク質の有意な変動を認めた。以上のことから，2種類の代表的な駆瘀血薬は，一部異なる作用機序で血流改善に影響を及ぼし，発現するタンパク質にも差異が認められた。作用機序とタンパク質発現との関連は今後検討を要するが，これらの多成分系の方剤による生体の複雑な反応性の差異が「証」の成立に影響していると考えられた。

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