

Inhibition of cardiac hypertrophy by probiotic-fermented purple sweet potato yogurt in spontaneously hypertensive rat hearts

PEI-PEI LIN¹, YOU-MIIN HSIEH¹, WEI-WEN KUO², CHIEN-CHUNG LIN³, FUU-JEN TSAI^{4,5},
CHANG-HAI TSAI⁶, CHIH-YANG HUANG^{4,7,8} and CHENG-CHIH TSAI⁹

¹Department of Food and Nutrition, Providence University; ²Department of Biological Science and Technology, China Medical University; ³Orthopaedic Department, Armed Forces General Hospital; ⁴Graduate Institute of Chinese Medical Science and ⁵Department of Pediatrics, Medical Research and Medical Genetics, China Medical University; Departments of ⁶Healthcare Administration and ⁷Health and Nutrition Biotechnology, Asia University; ⁸Graduate Institute of Basic Medical Science, China Medical University; ⁹Department of Food Science and Technology, HungKuang University, Taichung, Taiwan, R.O.C.

Received April 18, 2012; Accepted July 3, 2012

DOI: 10.3892/ijmm.2012.1154

Abstract. Cardiovascular hypertrophy is a common feature of hypertension and an important risk factor for heart damage. The regression of cardiovascular hypertrophy is currently considered an important therapeutic target in reducing the complications of hypertension. The aim of this study was to investigate the inhibition of cardiac hypertrophy by probiotic-fermented purple sweet potato yogurt (PSPY) with high γ -aminobutyric acid (GABA) content in spontaneously hypertensive rat (SHR) hearts. Six-week-old male SHRs were separated randomly and equally into 4 experimental groups: sterile water, captopril and 2 PSPY groups with different doses (10 and 100%) for 8 weeks. The changes in myocardial architecture and key molecules of the hypertrophy-related pathway in the excised left ventricle from these rats were determined by histopathological analysis, hematoxylin and eosin staining and western blot analysis. Abnormal myocardial architecture and enlarged interstitial spaces observed in the SHRs were significantly decreased in the captopril and PSPY groups compared with the sterile water group. Moreover, the increases in atrial natriuretic peptide, B-type natriuretic peptide, phosphorylated protein kinase Ca and calmodulin-dependent protein kinase II levels in the left ventricle were accompanied by hypertension and increases in phosphorylated extracellular signal-regulated kinase 5 activi-

ties with enhanced cardiac hypertrophy. However, the protein levels of the hypertrophic-related pathways were completely reversed by the administration of PSPY. PSPY may repress the activation of ANP and BNP which subsequently inhibit the dephosphorylation of the nuclear factor of activated T-cells, cytoplasmic 3 and ultimately prevent the progression of cardiac hypertrophy.

Introduction

Pathological cardiac hypertrophy is a common heart disease occurring in 20-50% of patients with mild to moderate hypertension and up to 90% of patients with severe hypertension (1). Although cardiac hypertrophy represents an adaptive response, prolonged hypertrophic status has been reported to be associated with decompensation of heart function, development of heart failure and sudden death in humans (2). Both conditions are independent risk factors for morbidity and mortality (3). The regression of cardiovascular hypertrophy is currently regarded as an important therapeutic target in reducing complications of hypertension (4).

The angiotensin-II-regulated downstream insulin-like growth factor II receptor (IGF-IIR) signaling pathway plays an important role in regulating the development of cardiac hypertrophy (5,6). Insulin-like growth factor II (IGF-II) is synthesized in most mammalian tissues. When IGF-II activates the IGF-IIR signaling pathway, IGF-II may activate calcineurin through Gi protein signaling transduction and stimulate cardiac cell hypertrophy (6). An additional pathway that has received attention is mediated by Ca²⁺-calmodulin activated phosphatase calcineurin. Once activated, calcineurin directly binds to and dephosphorylates the nuclear factor of the activated T-cells, cytoplasmic 3 (NFATc-3) transcription factor in the cytoplasm, permitting its translocation to the nucleus where dephosphorylated NFATc-3 further interacts with the GATA-4 transcription factor to form the complex that participates in the development of concentric hypertrophy and in the expression levels of hypertrophy responsive genes,

Correspondence to: Dr Cheng-Chih Tsai, Department of Food Science and Technology, HungKuang University, 34 Zhongqi Road, Shalu, Taichung 43302, Taiwan, R.O.C.
E-mail: tsaicc@sunrise.hk.edu.tw

Professor Chih-Yang Huang, Graduate Institute of Basic Medical Science, China Medical University and Hospital, 91 Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C.
E-mail: cyhuang@mail.cmu.edu.tw

Key words: cardiac hypertrophy, lactic acid bacteria, hypertension, fermented yogurt, γ -aminobutyric acid

such as atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) (7,8).

γ -aminobutyric acid (GABA) is a non-protein amino acid widely distributed in nature from microorganisms to plants and animals. It has several physiological functions, such as neurotransmission and the induction of hypotensive diuretic and tranquilizer effects (9-11). GABA may be obtained from a number of fruits and vegetables, but it is scarce in nature ranging from 0.03 to 2.00 $\mu\text{mol/g}$ fresh weight (12). GABA is synthesized by glutamate decarboxylase (GAD; EC 4.1.1.15), an enzyme that catalyzes the irreversible decarboxylation of L-glutamate to GABA. It has been reported that GAD is present in the mammalian brain, plants (13,14) and in lactic acid bacteria (LAB) (11). Therefore, many studies have focused on GABA production by using LAB as bacterial cell factories (10,12,15). The consumption of GABA-enriched foods has been reported to depress the elevation of systolic blood pressure in spontaneously hypertensive rats (SHRs) and mildly hypertensive humans (16,17). Vascular hypertrophy of the thoracic aorta, coronary and renal interlobular arteries has been shown to be reduced in SHRs with the oral administration of GABA-rich soy sauce, which is produced from moromi fermented with *Lactobacillus rennini* (18).

The purple sweet potato [*Ipomoea batatas* (L.) Lam.] (PSP) can be easily grown in tropical areas, such as Taiwan, Japan and China. It is rich in vitamins, minerals, dietary fiber and non-fibrous carbohydrates, as well as an excellent source of the antioxidant, anthocyanin (19,20). The aqueous extracts of anthocyanin-producing sweet potato have higher antiproliferative and antimutagenic potential than other crops (21), in which the bioactive compounds provide sensorial characteristics and are involved in cardiovascular disease risk protection (22). Previous studies have shown that treatment with antioxidants inhibits the hypertrophic response of cardiac myocytes (23-25). Thus, PSP may be used in the food industry as an antioxidant to improve human health.

In our previous study, we found that purple sweet potato yogurt (PSPY) fermented with *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *lactis* and *L. gasseri* has high GABA activity (26). To the best of our knowledge, previous studies regarding the potential role of GABA-enriched yogurt in hypertension-induced cardiac hypertrophy have not been conducted. Therefore, the purpose of this study was to determine the potency of PSPY to attenuate cardiac hypertrophy in SHRs and elucidate the anti-hypertrophic effects of PSPY on the intracellular transduction pathways in heart tissue *in vivo*. We anticipated that PSPY would be capable of providing dietary assistance in regulating blood pressure and preventing the development of cardiac hypertrophy.

Materials and methods

Bacteria strains and their growth conditions. The 3 LAB strains were purchased from the Food Industry Research and Development Institute of Biological Resources Conservation and Research Center, Hsinchu, Taiwan: *L. acidophilus* BCRC 14065 (LA), *L. delbrueckii* subsp. *lactis* BCRC 12256 (LDL) and *L. gasseri* BCRC 14619 (LGA). The stock culture was maintained at -80°C in 20% glycerol prior to usage. The bacteria were propagated twice in *Lactobacilli* MRS Broth

(DIFCO, Baltimore, MD, USA) containing 0.05% L-cysteine overnight at 37°C before experimental procedure.

Preparation of PSPY. The PSPs were acquired from the Taiwan Agricultural Research Institute and stored at 4°C after being washed with tap water. To make PSPY, the potatoes were first peeled, cut into 1-cm slices and steamed at 100°C for 20 min. The cooked spuds were then homogenized and before pasteurization (121°C , 15 min), a mixture of 0.05% α -amylase, 10% skimmed milk powder, 0.05% protease and 3% whey protein was added. The 3 different LAB strains were inoculated to the PSP milk and incubated at 37°C for 24 h until the fermented PSPY was obtained. The final product was stored at 4°C in the refrigerator for later experimental usage.

Animals and experimental groups. Twenty-two male SHRs and 12 male Wistar-Kyoto rats (WKYs) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). These animals, aged 6 weeks, were housed individually in a temperature ($20\pm 2^{\circ}\text{C}$)- and humidity ($55\pm 5\%$)-controlled environment. The rats were maintained on a 12-h dark-light cycle with lights on from 8 a.m. to 8 p.m. They were fed with chow pellets (MF-18; Oriental Yeast Co., Ltd., Tokyo, Japan) and were allowed access to water *ad libitum*. An acclimatization period of 1 week after delivery by the supplier was allowed before the SHRs were randomly distributed into 4 groups: SHR control (2.5 ml distilled water), antihypertensive captopril medicine (15.6 mg/kg, body weight/day), 10% PSPY (150 $\mu\text{g}/2.5$ ml) and 100% PSPY (1500 $\mu\text{g}/2.5$ ml). The WKY rats were used as the negative control. The rats were sacrificed after 8 weeks of the experimental period. The entire experimental procedure was performed according to the NIH Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use Committee of Hungkuang University, Taichung, Taiwan (approval no. 96027).

Body weight and cardiac characteristics. The rats were weighed first and then sacrificed by decapitation. The hearts of the rats were removed and cleaned with double distilled H_2O before dehydration. The left and right atrium and ventricle were separated. The dry weight of the whole heart and left ventricle was obtained, and ratios of the 2 measurements to rat body weight plus the ratio of left ventricle weight to the whole heart weight were calculated independently.

Cross-section and hematoxylin and eosin (H&E) staining. The heart was soaked in formalin and covered with wax after removal. Cross-sections of the whole heart were sliced and the maximal cross-section was selected. Slides were prepared by first soaking them for dehydration. They were passed through a series of graded alcohols (100, 95 and 75%), 15 min for each. The slides were dyed with Mayer's hematoxylin for 5-10 min and then washed with tap water for 10-20 min. Each slide was soaked in mild warm water until it turned bright violet and then placed into eosin solution for 3-5 min. After gently rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol I and II for 15 min each. Finally, each slide was soaked with Xylene I-Xylene II. Photomicrographs were obtained using Zeiss Axiophot microscopes (magnification, x200) (Olympus, Japan).

Table I. Cardiac characteristics of WKYs and SHR treated with captopril and PSPY.

No. of animals	WKYs		SHRs		
	Control (n=10)	Control (n=3)	Captopril (n=4)	10% PSPY (n=3)	100% PSPY (n=4)
Body weight (BW), g	302.20±11.71	287.33±15.54	279.00±16.53	303.33±23.86	296.50±15.52
Whole heart weight (WHW), g	1.11±0.28 ^b	1.24±0.04 ^a	1.12±0.01 ^b	1.24±0.05 ^a	1.23±0.05 ^a
Left ventricle weight (LVW), g	0.81±0.21 ^b	1.02±0.02 ^a	0.91±0.05 ^b	1.00±0.03 ^a	1.02±0.03 ^a
WHG/BW x10 ³	3.66±0.91 ^b	4.32±0.21 ^a	4.01±0.20	4.09±0.17 ^a	4.14±0.09 ^a
LVW/BW x10 ³	2.67±0.70 ^b	3.55±0.12 ^a	3.28±0.23 ^a	3.29±0.22 ^a	3.44±0.12 ^a
LVW/WHW	0.73±0.04 ^b	0.82±0.02 ^a	0.82±0.05 ^a	0.80±0.02	0.83±0.01 ^a

Values are expressed as the means ± standard deviation (SD). ^aP<0.05, compared with the WKY group; ^bP<0.05, compared with the SHR-control group. WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat; PSPY, purple sweet potato yogurt.

Tissue extraction. The left ventricle was cut into 8 parts. One part of the left ventricle was minced with scissors, added to lysis buffer (20 mM Tris, 2.0 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, pH 7.4), proteinase inhibitor cocktail tablet and phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at a concentration of 100 mg tissue/ml buffer and homogenized at ice temperature with a Model PT 10/35 Polytron homogenizer for 2 cycles of 10 sec each. The homogenate was placed on ice for 10 min and then centrifuged at 12,000 x g for 40 min. The supernatant was collected and stored at -70°C for further western blot analysis.

Protein contents. The protein contents of the left ventricle extract were determined using the Bradford protein assay 14 using the protein-dye kit (Bio-Rad, Hercules, CA, USA). A commercially available bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) was used as the standard. Changes in absorption were monitored at 595 nm.

Electrophoresis and western blot analysis. The left ventricle extract samples were prepared as described above. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed using 10% polyacrylamide gels. Equal amounts (20 mg) of the samples were electrophoresed at 100 V for 3 h and equilibrated for 15 min in transfer buffer [25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol]. The electrophoresed proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, 0.45 µm pore size) using a Bio-Rad Scientific Instruments Transphor Unit at 100 V with transfer buffer for 3 h. PVDF membranes were incubated at room temperature for 1 h in the blocking buffer containing 100 mM Tris-Base, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 (pH 7.4) and 5% non-fat milk. Monoclonal antibodies of protein kinase Ca (PKCα), Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), phosphorylated extracellular signal-regulated kinase 5 (p-ERK5) (Millipore-Upstate, Billerica, MA, USA), calcineurin (BD Pharmingen, San Diego, CA, USA) and polyclonal antibodies of ANP, BNP, IGF II, phosphorylated PKCα (p-PKCα), guanine nucleotide-binding protein G(q) subunit α (Gαq), phosphorylated NFATc3 (p-NFATc3) and interleukin 6

(IL-6) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were diluted in an antibody-binding buffer containing 100 mM Tris-Base, pH 7.5, 0.9% (w/v) NaCl and 0.1% (v/v) Tween-20. The immunoblots were washed 3 times in binding buffer for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.) for 1 h and diluted 500-fold in binding buffer. The filters were then washed 3 times (10 min each) in blotting buffer. The immunoblotted proteins were visualized using an enhanced chemiluminescence ECL western blotting luminol reagent (Santa Cruz Biotechnology, Inc.) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan). The color was developed in a 20 ml mixture consisting of 7 mg nitro blue tetrazolium, 5 mg 5-bromo-4-chloro-3-indolyl-phosphate, 100 mM NaCl and 5 mM MgCl₂ in 100 mM Tris-HCl, pH 9.5. The immunoblot with antibody against α-tubulin, which was prepared with the same procedure, was used as the internal control.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The data were compared between groups of animals, using one-way analysis of variance (ANOVA). Dunnett's test was used to determine significant differences. P-values <0.05 were considered to indicate statistically significant differences. The significant differences are indicated with symbols as shown in the tables and figures.

Results

Body weight and cardiac characteristics. There was no significant difference in body weight (P<0.05) among the SHR-control, SHR-captopril, SHR-PSPY (both doses, 10 and 100%) and WKY groups (Table I). Whole heart weight, left ventricular weight, the ratio of the whole heart weight to body weight, left ventricular weight to body weight and left ventricular weight to whole heart weight were significantly higher in the SHR-control and SHR-PSPY (both doses, 10 and 100%) groups, whereas the whole heart weight and left ventricular weight were lower in the SHR-captopril group,

Table II. Cardiac characteristics by tissue cross-sections of WKYs and SHRs with captopril and PSPY.

No. of animals	WKY	SHR			
	Control (n=3)	Control (n=3)	Captopril (n=3)	10% PSPY (n=3)	100% PSPY n=3
Diameter of LV (mm)	0.98±0.02	0.94±0.01	1.03±0.01 ^{a,d}	1.04±0.04 ^c	0.91±0.01 ^{b,c}
Thickness of LV (mm)	0.45±0.01	0.44±0.01	0.46±0.01	0.48±0.02	0.39±0.02 ^{b,c}
Thickness/diameter (mm)	0.46±0.01	0.47±0.01	0.45±0.01	0.46±0.01	0.43±0.01 ^{a,c}

Values are mean ± SD. LV, left ventricle. ^aP<0.05, ^bP<0.01, compared with the WKY group; ^cP<0.05, ^dP<0.01, compared with the SHR-control group. WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat; PSPY, purple sweet potato yogurt.

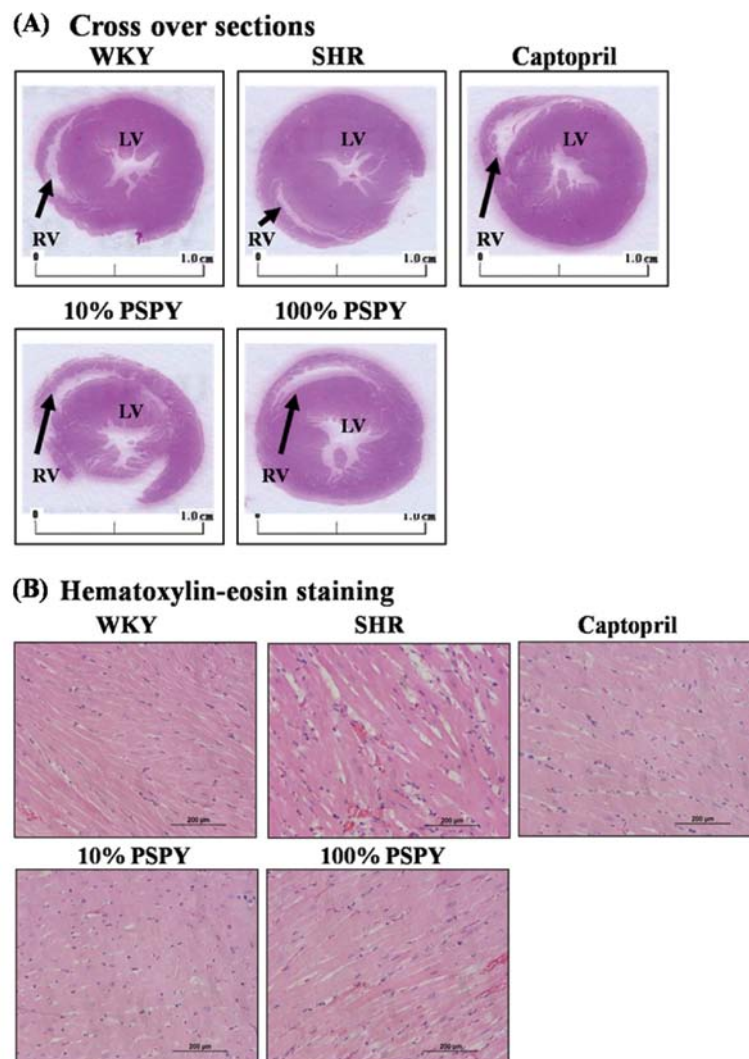


Figure 1. Representative histopathological analysis of cardiac tissue sections. (A) Cross sections of hearts; (B) H&E staining in normal control rats (Wistar-Kyoto rats, WKY group), spontaneously hypertensive rats (SHR group), different doses of PSPY (10 and 100% PSPY groups). RV, right ventricle; LV, left ventricle. The images of the myocardial architecture were observed under magnification, x200.

compared with those of the WKY normal controls. However, the ratio of the whole heart weight to the body weight, which was traditionally regarded as an index of cardiac hypertrophy, was lower both in the SHR-captopril, SHR-10 and 100% PSPY groups than in the SHR-control group (Table I).

Cardiac architectural changes. To further define the characteristics of cardiac hypertrophy, we made a cross-section of the whole heart and carried out a histopathological analysis of the ventricular tissue stained with H&E. We found that the SHR controls presented no significant difference in ventricular wall

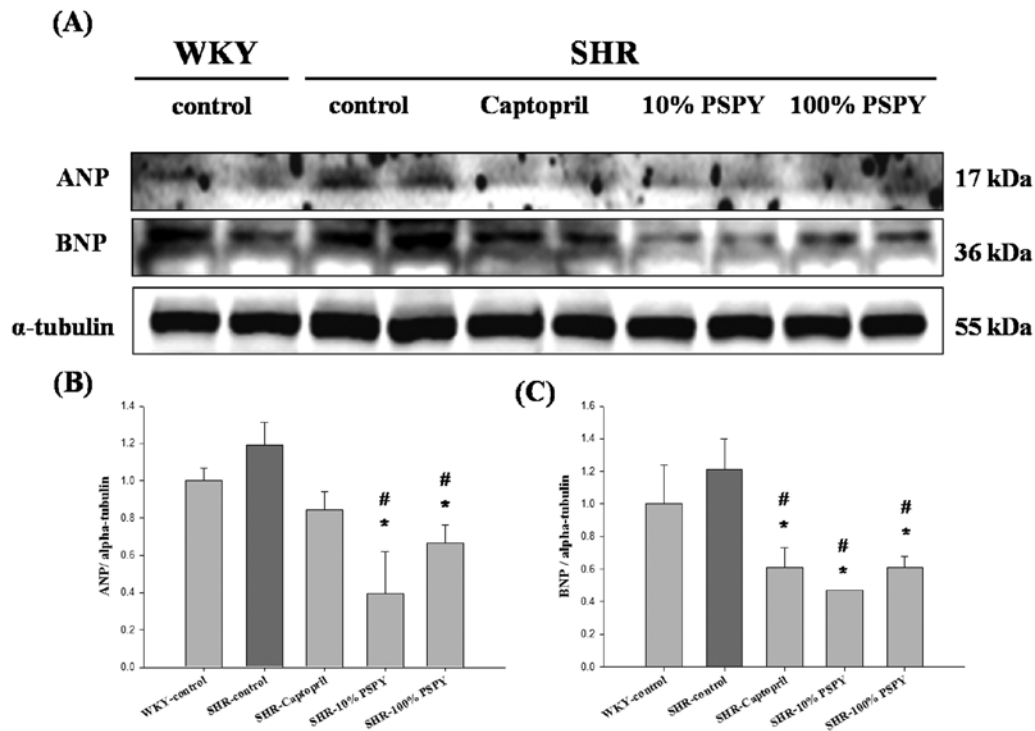


Figure 2. (A) The protein products of ANP and BNP extracted from the left ventricles of excised hearts in the WKY, SHR, SHR-10% and 100% PSPY groups were measured by western blot analysis. (B and C) Bars represent the relative protein quantification of ANP and BNP on the basis of α -tubulin, and indicate the mean values \pm SD. (n=3 in each group). *P<0.05, compared with the WKY group. #P<0.05, compared with the SHR-control group.

thickness and in the ratio of wall thickness to cavity diameter compared to the WKY control group. However, the ventricular wall thickness was significantly reduced (P<0.05 and 0.01) only in the SHR-100% PSPY group, compared with those of the SHR-control and WKY-control groups (Table II and Fig. 1A). Similarly, the ratio of wall thickness to cavity diameter was significantly (P<0.05) decreased in the SHR-100% PSPY group (Table II and Fig. 1A). Although the SHR-captopril and 10% PSPY groups presented no significant difference in either ventricular wall thickness or ratio of wall thickness to cavity diameter compared to the control groups, the SHR-captopril and 10% PSPY groups had an enhanced diameter of the left ventricle. Moreover, the ventricular myocardium in the WKY group showed normal architecture with normal interstitial space. By contrast, abnormal myocardial architecture, such as cardiomyocyte disarray and increased interstitial space were observed in the SHR-control group (Fig. 1B). Nevertheless, restorations of myocardial architecture were observed in the SHR-captopril, SHR-10% and 100% PSPY groups, presenting a normal interstitial space as observed in the WKY control group (Fig. 1B).

Changes in ANP and BNP pathological hypertrophy markers of the rat left ventricle. To further investigate whether the proteins associated with cardiac pathological hypertrophy are influenced by PSPY, the pathological hypertrophy markers such as ANP and BNP in the left ventricle were analyzed by western blotting. The levels of ANP and BNP, markers of left ventricular remodeling associated with cardiac hypertrophy, were higher in the SHR-control than in the WKY group (Fig. 2), without reaching significant levels. Moreover, ANP

and BNP protein levels were lower in the SHR-10% and 100% PSPY groups than in the SHR-control rats (P<0.05) (Fig. 2B and C).

Changes in IGF-II pathway-related proteins of the rat left ventricle. To investigate the hypertrophic factor, IGF-II, and its cardiac hypertrophy-associated downstream signaling pathway that was influenced by PSPY, the protein products of IGF-II were measured by western blot analysis. However, the protein products of IGF-II were lower in the SHR-captopril, 10% and 100% PSPY groups, compared with the SHR-control, although all the SHR groups showed no significant difference compared to the WKY group (Fig. 3B). The downstream signaling pathway IGF-II-related protein levels, including those of G α q, PKC α , p-PKC α and CaMKII were significantly increased in the SHR-control compared to the WKY group (P<0.05) (Figs. 3C, 4 and 5). Compared with the SHR-control group, the levels of the p-PKC α and CaMKII proteins were decreased significantly in the SHR-captopril, 10% and 100% PSPY groups (P<0.05) (Figs. 4C and 5B). The PKC α protein level was decreased significantly in the SHR-captopril and 100% PSPY groups compared to the SHR group.

Changes in calcineurin/NFATc-3 pathway proteins of the rat left ventricle. The results summarized in Figs. 3-5 suggest that PSPY regulates the levels of the IGF-II- and downstream-related proteins of myocardial hypertrophy in SHR. Therefore, we used western blot analysis to extend the analysis on calcineurin and NFATc-3, which are both indicators of the development of cardiac hypertrophy. In the calcineurin/NFATc-3 pathway, the calcineurin protein levels

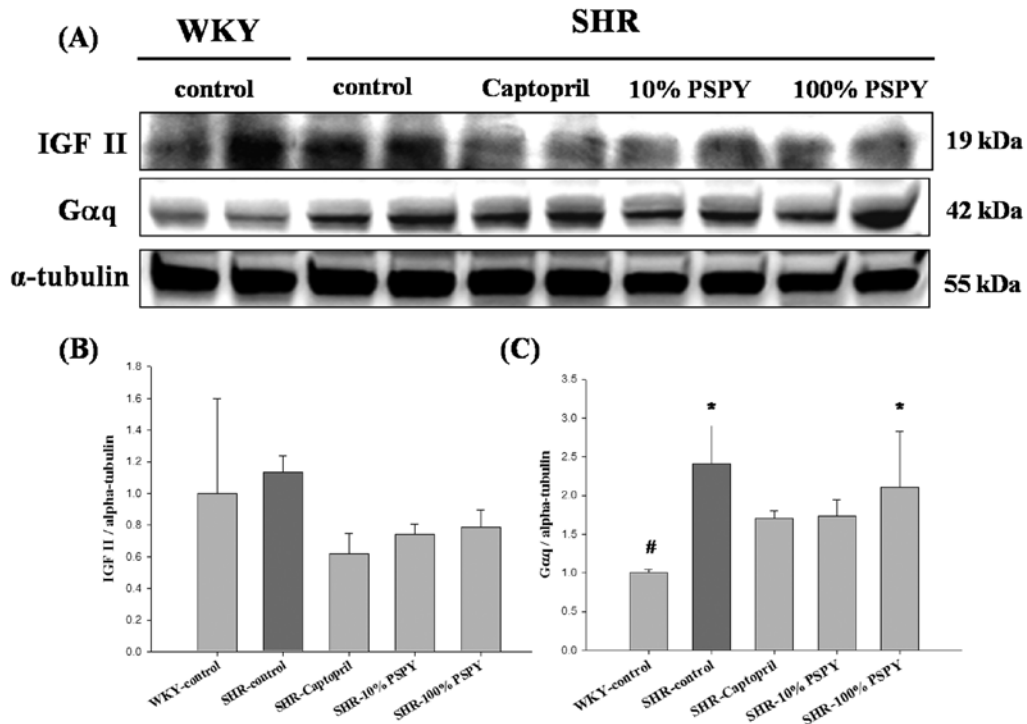


Figure 3. (A) The protein products of IGF-II and Gαq extracted from the left ventricles of excised hearts in the normal control rats WKY, SHR , SHR-10% and 100% PSPY groups were measured by western blot analysis. (B and C) Bars represent the relative protein quantification of Gαq and IGF II on the basis of α-tubulin, and indicate the mean values ± SD. (n=3 in each group). *P<0.05, compared with the WKY group. #P<0.05, compared with the SHR-control group.

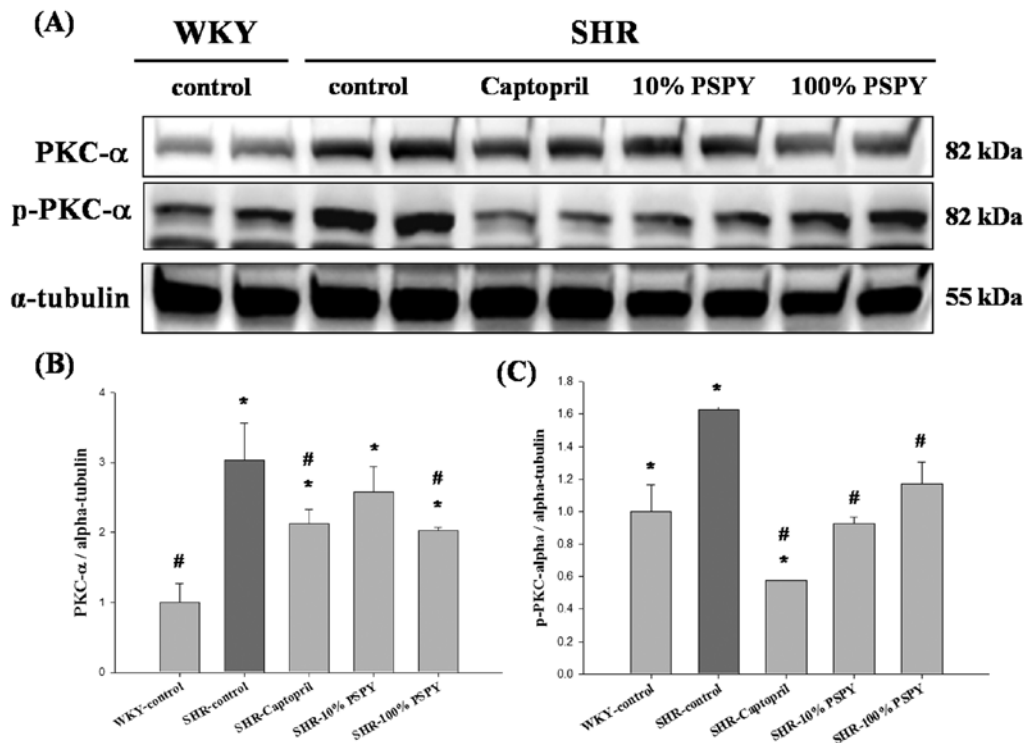


Figure 4. (A) The protein products of PKCα and p-PKCα extracted from the left ventricles of the excised hearts in the WKY, SHR, SHR-10% and 100% PSPY groups were measured by western blot analysis. (B and C) Bars represent the relative protein quantification of PKCα and p-PKCα on the basis of α-tubulin, and indicate the mean values ± SD. (n=3 in each group). *P<0.05, compared with the WKY group. #P<0.05, compared with the SHR-control group.

were slightly lower in the WKY and SHR-captopril groups than in the SHR-control group (Fig. 6A), whereas no changes

in the SHR-10% and 100% PSPY groups were observed. However, the p-NFATc-3 levels were significantly decreased

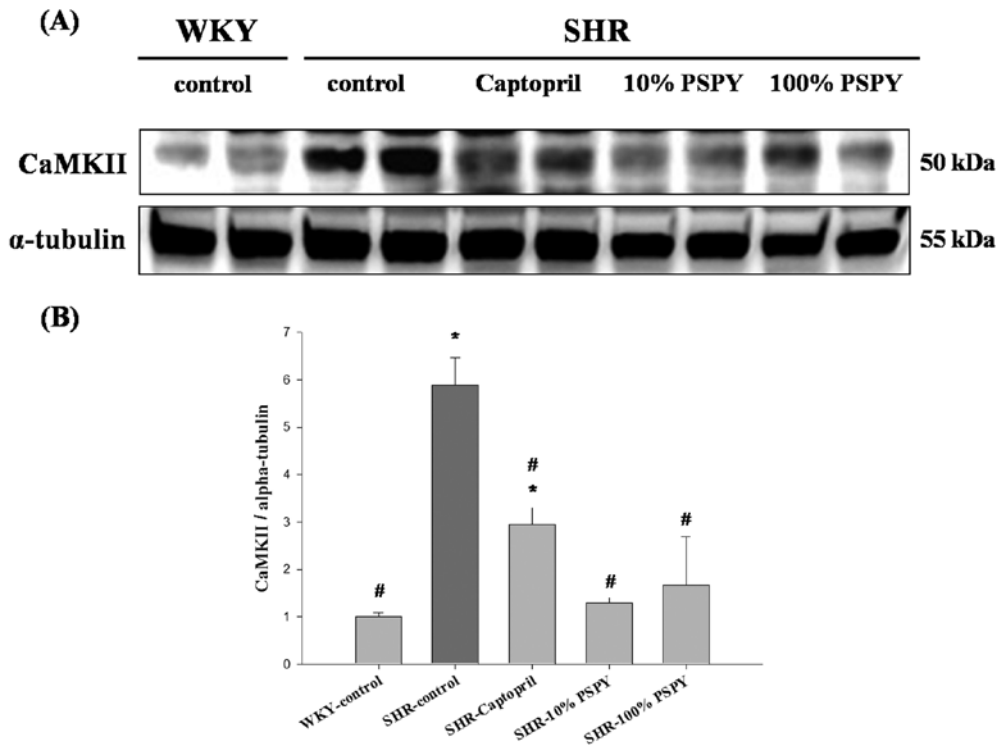


Figure 5. (A) The protein products of CaMKII extracted from the left ventricles of excised hearts in the WKY, SHR, SHR-10% and 100% PSPY groups were measured by western blot analysis. (B) Bars represent the relative protein quantification of CaMKII on the basis of α -tubulin, and indicate the mean values \pm SD. (n=3 in each group). *P<0.05, compared with the WKY group. #P<0.05, compared with the SHR-control group.

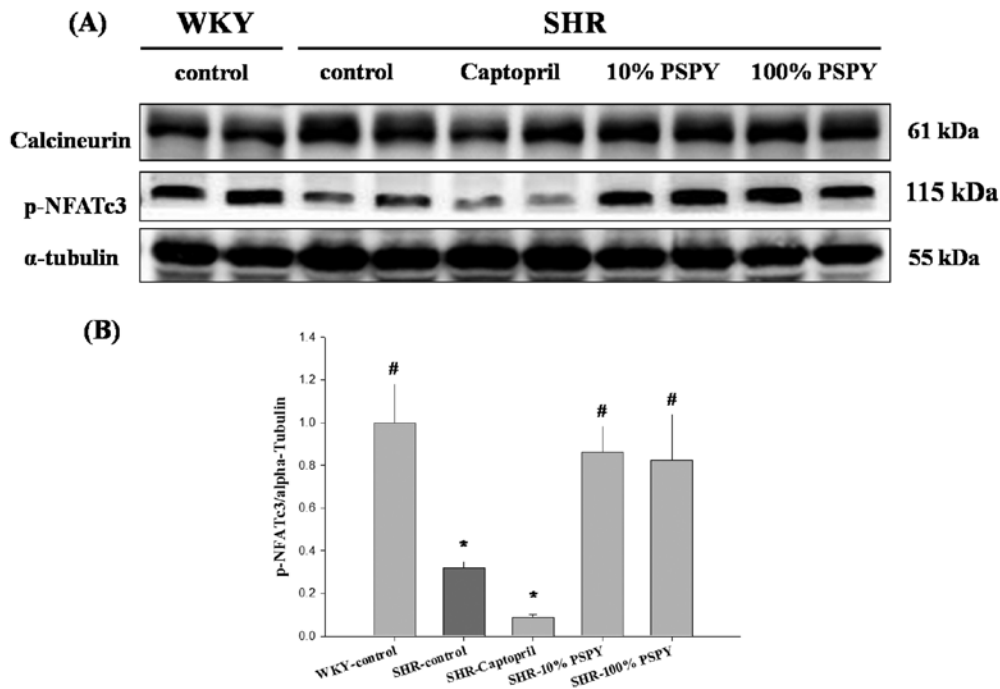


Figure 6. (A) The protein products of calcineurin and p-NFATc3 extracted from the left ventricles of the excised hearts in the WKY, SHR, SHR-10% and 100% PSPY groups were measured by western blot analysis. (B) Bars represent the relative protein quantification of p-NFATc3 on the basis of α -tubulin, and indicate the mean values \pm SD. (n=3 in each group). *P<0.05, compared with the WKY group. #P<0.05, compared with the SHR-control group.

in the SHR-control group compared with the WKY group, but increased both in the SHR-10% and 100% PSPY groups (Fig. 6). Quantitative results of the p-NFATc-3 protein in both PSPY groups were normalized close to the levels presented

in the WKY group (P<0.05) (Fig. 6B). These results suggest that PSPY affects ANP and BNP protein levels by inhibiting the dephosphorylation of NFATc-3 and therefore impedes the development of myocardial hypertrophy.

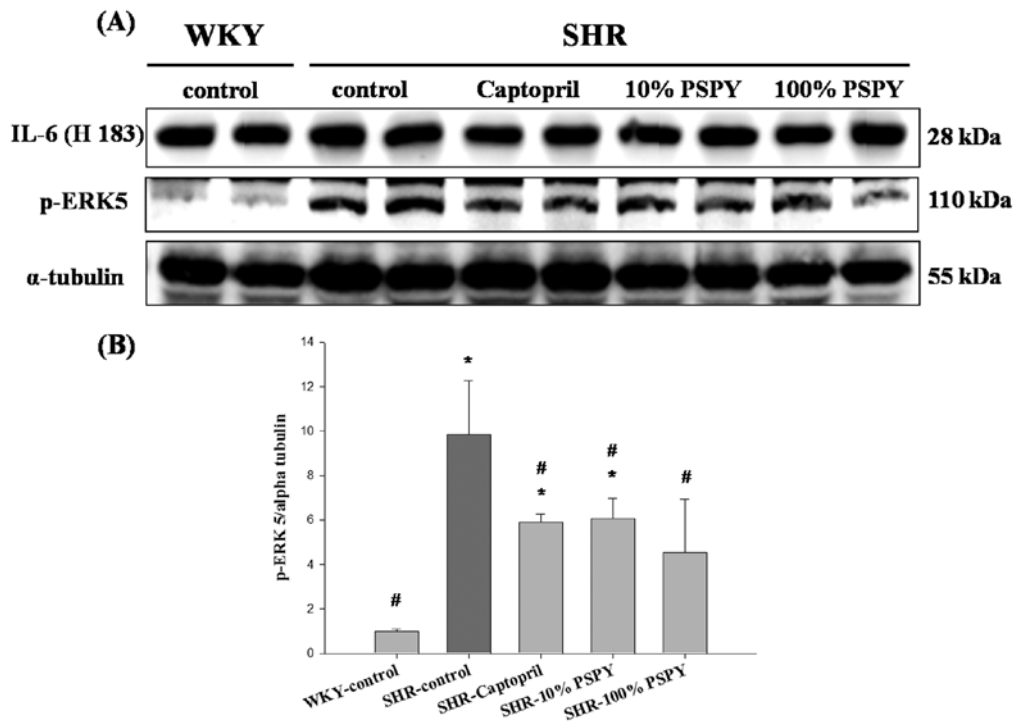


Figure 7. (A) The protein products of IL-6 and p-ERK5 extracted from the left ventricles of the excised hearts in the WKY, SHR, SHR-10% and 100% PSPY groups were measured by western blot analysis. (B) Bars represent the relative protein quantification of p-ERK5 on the basis of α -tubulin, and indicate the mean values \pm SD. (n=3 in each group). *P<0.05, compared with the WKY group. #P<0.05, compared with the SHR-control group.

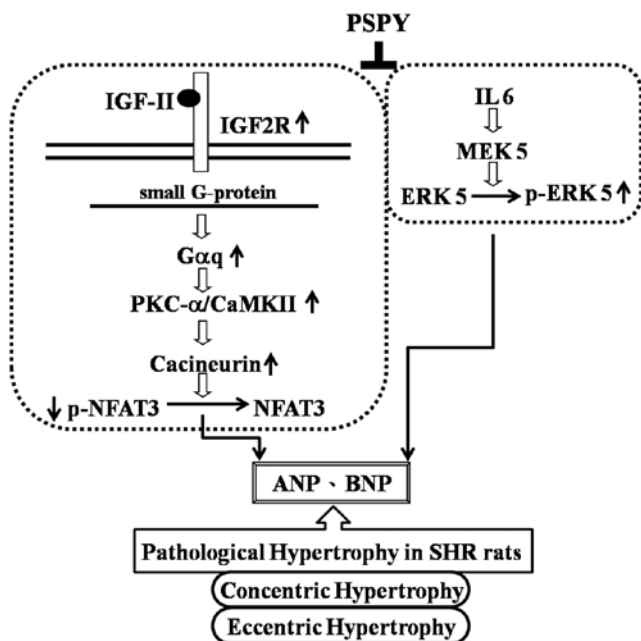


Figure 8. Schematic diagram showing that PSPY inhibits hypertrophy in SHRs through intracellular IGF-IIR and IL-6 signaling suppression.

Changes in IL-6 and p-ERK5 protein levels of the rat left ventricle. In order to identify the hypertrophic factor, IL-6, and cardiac hypertrophy-associated mitogen-activated protein kinase 5 (MEK5)/ERK5 signaling pathways that were influenced by PSPY, the protein products of IL-6 and p-ERK5 were measured by western blot analysis (Fig. 7). The protein

level of IL-6 was not significantly different in all SHR groups compared to the WKY group, whereas p-ERK5 was increased markedly in the SHR-control group and significantly decreased in the SHR-captopril, 10% and 100% PSPY groups, when compared to the WKY group (P<0.05) (Fig. 7B).

Discussion

The main findings of the present study may be summarized as follows: i) the ratio of the whole heart weight to body weight and myocardial architecture with the interstitial space were increased in the SHRs that were associated with significantly increased protein levels of Gaq, PKC α , p-PKC α , CaMKII, as well as p-ERK5 and decreased protein levels of p-NFATc3; ii) the SHR-captopril and SHR-PSPY (both doses, 10 and 100%) groups showed normal myocardial architecture with normal interstitial space similar to the WKY controls and a decreased ratio of whole heart weight to body weight. Furthermore, the pathological hypertrophy markers, ANP and BNP, as well as the protein levels of p-PKC α , CaMKII and p-ERK5 were decreased. However, the SHR-PSPY group had a higher level of p-NFATc3 similar to that in the WKY controls. iii) In conclusion, we hypothesized that hypertension caused cardiac pathological hypertrophy that was associated with the increase in the levels of the hypertrophic factors, ANP, BNP and IGF-II, and the signaling molecules, Gaq, PKC α , p-PKC α , CaMKII. However, the captopril and PSPY groups may suppress the hypertrophic factors and pathological hypertrophic effect. Moreover, PSPY inhibited both the concentric and eccentric hypertrophy determining molecules and enhanced the p-NFATc-3 and reduced p-ERK5 protein levels (Fig. 8).

Pathological cardiac hypertrophy is associated with ventricular remodeling through alterations in the extracellular matrix that eventually impact the cardiac function and energy utilization (27) and increase the myocyte cell death rate through apoptotic and necrotic mechanisms (28). Hypertrophy is associated with disease-inducing stimuli, such as chronic hypertension that may produce concentric hypertrophy in which the ventricular wall and septum thicken with a net decrease in ventricular chamber dimensions. This remodeling is associated with a greater increase in cardiac myocyte width than length. In addition, pathological cardiac hypertrophy may also produce a phenotype of eccentric and dilatory cardiac growth. Cardiac dilation, although not typically referred to as hypertrophy, may result from a growth response in which sarcomeres are predominantly added in a series to individual myocytes (29). Therefore, cardiac hypertrophy may be characterized by quantitative effects on cell size and width.

We observed that the captopril, 10 and 100% PSPY groups had reduced cardiac hypertrophy index and whole heart weight to body weight ratio. In addition, the ventricular wall thickness and wall thickness to cavity diameter ratio were significantly suppressed in the 100% PSPY group. The captopril and 10% PSPY groups had an enhanced diameter of the left ventricle. Moreover, an abnormal myocardial architecture and increased interstitial space were observed in the SHR; however, the PSPY groups (10 and 100% doses) appeared more normal in architecture. Similarly, a recent study showed that *L. paracasei* NTU 101 (101FM) and *L. plantarum* NTU 102 fermented milk had antihypertensive effects, possibly due to ACEI and GABA activities in SHRs, and also reduced the disorganization of the aortic media layer (30). Liu *et al* (30) suggested that these hypotensive effects depend on decreased peripheral vascular resistance. Accordingly, we speculated that hypertension could induce cardiac hypertrophy and abnormal myocardial architecture. However, captopril and PSPY may somewhat ameliorate this situation. Further investigation on the mechanisms of the involved proteins influenced by PSPY in hypertrophic signaling pathways is required.

ANP and BNP are circulating hormones of cardiac origin that play an important role in regulating the permeability of systemic vasculature, cellular growth, cellular proliferation and, as previously recently, cardiac hypertrophy (31). In a previous study conducted by Chu *et al* (32), treatment with angiotensin-II and IGF-II induced H9c2 cardiomyoblast cell hypertrophy and an increase in the protein levels ANP and BNP (6). As shown in Fig. 2, PSPY inhibited NFATc3 activation which subsequently repressed the reactivation of ANP and BNP in hypertensive rats. These observations indicate that PSPY has a positive effect on reducing the levels of protein markers of cardiac hypertrophy.

Previous studies have shown that IGF II directly induces the hypertrophy of adult rat ventricular cardiomyocytes in serum-free medium, as demonstrated by their increased size, total protein synthesis and transcription of muscle-specific genes (5), suggesting that IGF-II and IGF-II-mediated pathways are possibly involved in the IGF-IIR pathway and stimulate the hypertrophy of cardiomyocytes (6,33). Moreover, specifically-activated IGF2R signaling by Leu27IGF-II has been shown to trigger the phosphorylation of PKC α /CaMKII signaling in order to induce cell hypertrophy (32). Although

IGF-II and G α q were not significantly increased in all the SHR groups compared to the WKY group, the downstream IGF-II signaling pathway protein levels, including those of p-PKC α and CaMKII were significantly increased in the hypertensive rats. However, we found that p-PKC α /CaMKII through G α q were activated in the hypertensive rats and were inhibited by the administration of captopril and PSPY.

Calcineurin is a serine/threonine-specific phosphatase activated by a sustained increase in calcium. A large number of studies have demonstrated that the calcineurin/NFATc-3 signaling pathway plays an important role in the development of cardiac hypertrophy. Calcineurin directly binds to and dephosphorylates multiple conserved serine residues in the N-terminus of NFAT-3 transcription factors, permitting their translocation to the nucleus and promoting the subsequent induction of the hypertrophic gene program (7,34). It has also been demonstrated that calcineurin activity is increased in compensated hypertrophied human myocardium and end-stage heart failure (35). In the present study, we observed that the protein level of calcineurin was not reduced in the SHR-10% and 100% PSPY groups. However, the downstream phosphorylation of NFATc-3 was significantly decreased in the SHRs, but highly increased in both 10% and 100% PSPY groups. PSPY may regulate phosphorylated calcineurin and lead to the direct decrease in the nuclear localization of NFATc-3 in SHR hearts. The transcription factor of p-NFAT-3 increased in the cytoplasm in order to suppress the expressions of hypertrophy response markers, such as ANP and BNP.

Interleukin-6 (IL-6), a typical cytokine, was found to have a potent hypertrophic effect on cardiomyocytes (36). The concomitant overexpression of both IL-6 and the IL-6 receptor (IL-6R) in mice induces concentric hypertrophy typical of that occurring in a hypertensive heart. IL-6/IL-6R does this by interacting with a membrane-bound glycoprotein (gp130), which in turn leads to the phosphorylation of the downstream second messengers, such as Janus kinase, to induce signal transduction and activators of transcription, causing the stimulation of various cellular events (37). IL-6 is involved in multiple intracellular signaling pathways, including p38 MAPK, STAT-1-STAT-3 heterodimer, STAT-3 homodimer and ERKs pathways (38,39). The ERK5 and its upstream MAPK-kinase 5 (MEK5) reveal a specific role in the transduction of cytokine signals that regulate serial sarcomere assembly and play a role in the induction of eccentric cardiac hypertrophy that progresses to dilated cardiomyopathy and sudden death (40). The MEK5-ERK5 pathway plays a critical role in the induction of eccentric cardiac hypertrophy that progresses to dilated cardiomyopathy and sudden death (40,41). In the present study, we observed the IL-6 was not significantly different among all the SHR groups whereas p-ERK5 was highly activated in the SHR-control group, but significantly suppressed in the SHR-captopril, SHR-10 and 100% PSPY groups. These observations suggest that the inhibitory effect of PSPY on the activation of ERK5 is regulated by additional upstream messengers, such as Janus kinase or MEK5, rather than IL-6. Therefore, we strongly suggest that PSPY possibly via the ERK5 inactivation, blocks the IL-6-MEK5-ERK5 pathway-related eccentric dilated cardiac hypertrophic effects.

Our previous experimental data showed that the concentrations of GABA in PSPY with a mixture of 3 LAB strains (LA,

LGA and LDL) and a control (PSP without LAB strains) were $1068.8 \pm 21.3 \mu\text{g/ml}$ and $250.0 \pm 10.1 \mu\text{g/ml}$, respectively (26). The findings indicate that the probiotics fermentation process may enhance the production of GABA in PSPY. Our previous animal experimentation showed that continuous PSPY feedings for 8 weeks significantly reduced the blood pressure of SHR (data not shown). Interestingly, our results from histopathological analysis and analysis of key molecules of the cardiac hypertrophy-related intracellular signaling pathway demonstrated that the oral administration of low-dose PSPY (10% dosage) was sufficient in order to prevent cardiac hypertrophy. There was no significant dose-response effect observed between 10-fold differences in PSPY concentration (10 and 100%). The mechanisms underlying the low- and high-dose hypotensive effects of GABA may account for this difference in effect. The administration of GABA at a low dose was considered to decrease blood pressure through a peripheral mechanism (without crossing the brain blood barrier). However, a high dose of GABA is considered to occur via a central mechanism (16). The majority of efforts have concentrated on designing highly hydrophobic derivatives of GABA that may easily permeate brain tissue (42).

Other substances in PSPY may contribute to the anti-hypertrophic effects, such as antioxidant activity. Current research on the antioxidant ability of LAB has shown that some LAB strains cannot reduce the risk of reactive oxygen species accumulation through food ingestion but may degrade the superoxide anion and hydrogen peroxide (43). PSPs are also an excellent source of anthocyanins which account for their antioxidant activity. In our previous study, we indicated that the fermentation procedure may further elevate anthocyanin antioxidative activity significantly in PSPY with LAB strains (26). Li *et al.* (25) showed that the natural antioxidant, epigallocatechin-3-gallate (EGCG), inhibited angiotensin-II-induced NF- κ B and AP-1 activation, which subsequently repressed the reactivation of ANP and BNP, and ultimately prevented the progress of cardiac hypertrophy. Thus, the combination of these factors may result in interfering with the hypertrophy-related intracellular signaling pathway.

In conclusion, PSPY may mediate the anti-hypertrophic effect in the SHR hearts by interfering with different intracellular signaling pathways. The results from the present study may aid in the understanding of the effects of PSPY on cardiac hypertrophy and may shed light on the related molecular mechanisms. PSPY may also suppress cellular signaling associated with angiotensin-II-regulated IGF-II signaling pathways and the IL-6-related-ERK5 pathway and may provide a novel insight into the prevention and treatment of the pathological hypertrophic process. However, extensive clinical trials or studies *in vivo* on the human system are required to determine the long-term treatment effects of PSPY and the half-life period and optimum dosage for beneficial effects.

Acknowledgements

This study was supported in part by the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH101-TD-B-111-004), the Taiwan Department of Health Cancer Research Center of Excellence (DOH101-TD-C-111-005) and the National Science Council, Taiwan, R.O.C. (NSC97-2313-B-241-004-MY3).

References

1. Kuo WW, Chu CY, Wu CH, Lin JA, Liu JY, Hsieh YH, Ueng KC, Lee SD, Hsieh DJ, Hsu HH, *et al.*: Impaired IGF-I signalling of hypertrophic hearts in the developmental phase of hypertension in genetically hypertensive rats. *Cell Biochem Funct* 23: 325-331, 2005.
2. Towbin JA and Bowles NE: The failing heart. *Nature* 415: 227-233, 2002.
3. DeBlois D, Tea BS, Beaudry D and Hamet P: Regulation of therapeutic apoptosis: a potential target in controlling hypertensive organ damage. *Can J Physiol Pharmacol* 83: 29-41, 2005.
4. Kjeldsen SE, Dahlof B, Devereux RB, Julius S, Aurup P, Edelman J, *et al.*: Effects of losartan on cardiovascular morbidity and mortality in patients with isolated systolic hypertension and left ventricular hypertrophy: a Losartan Intervention for Endpoint Reduction (LIFE) substudy. *JAMA* 288: 1491-1498, 2002.
5. Huang CY, Hao LY and Buetow DE: Insulin-like growth factor-induced hypertrophy of cultured adult rat cardiomyocytes is L-type calcium-channel-dependent. *Mol Cell Biochem* 231: 51-59, 2002.
6. Lee SD, Chu CH, Huang EJ, Lu MC, Liu JY, Liu CJ, Hsu HH, Lin JA, Kuo WW and Huang CY: Roles of insulin-like growth factor II in cardiomyoblast apoptosis and in hypertensive rat heart with abdominal aorta ligation. *Am J Physiol Endocrinol Metab* 291: E306-E314, 2006.
7. Molkenin JD, Lu JR, Antos CL, *et al.*: A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 93: 215-228, 1998.
8. Wilkins B, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF and Molkenin JD: Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophy growth. *Mol Cell Biol* 22: 7603-7613, 2002.
9. Komatsuzaki N, Nakamura T, Kimura T and Shima J: Characterization of glutamate decarboxylase from a high gamma-aminobutyric acid (GABA)-producer, *Lactobacillus paracasei*. *Biosci Biotechnol Biochem* 72: 278-285, 2008.
10. Li H and Cao Y: Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino Acids* 39: 1107-1116, 2010.
11. Ueno Y, Hayakawa K, Takahashi S and Oda K: Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005. *Biosci Biotechnol Biochem* 61: 1168-1171, 1997.
12. Kim JY, Lee MY, Ji GE, Lee YS and Hwang KT: Production of gamma-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. *Int J Food Microbiol* 130: 12-16, 2009.
13. Denner LA and Wu JY: Two forms of rat brain glutamic acid decarboxylase differ in their dependence on free pyridoxal phosphate. *J Neurochem* 44: 957-965, 1985.
14. Oh SH, Choi WG, Lee IT and Yun SJ: Cloning and characterization of a rice cDNA encoding glutamate decarboxylase. *J Biochem Mol Biol* 38: 595-601, 2005.
15. Yokoyama S, Hiramatsu J and Hayakawa K: Production of gamma-aminobutyric acid from alcohol distillery lees by *Lactobacillus brevis* IFO-12005. *J Biosci Bioeng* 93: 95-97, 2002.
16. Hayakawa K, Kimura M, Kasaha K, Matsumoto K, Sansawa H and Yamori Y: Effect of a gamma-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Br J Nutr* 92: 411-417, 2004.
17. Inoue K, Shirai T, Ochiai H, Kasao M, Hayakawa K, Kimura M and Sansawa H: Blood-pressure-lowering effect of a novel fermented milk containing gamma-aminobutyric acid (GABA) in mild hypertensives. *Eur J Clin Nutr* 57: 490-495, 2003.
18. Yamakoshi J, Fukuda S, Satoh T, Tsuji R, Saito M, Obata A, Matsuyama A, Kikuchi M and Kawasaki T: Antihypertensive and natriuretic effects of less-sodium soy sauce containing gamma-aminobutyric acid in spontaneously hypertensive rats. *Biosci Biotechnol Biochem* 71: 165-173, 2007.
19. Philpott M, Lim CC and Ferguson LR: Dietary protection against free radicals: A case for multiple testing to establish structure-activity relationships for antioxidant potential of anthocyanic plant species. *Int J Mol Sci* 10: 1081-103, 2009.
20. Philpott M, Gould KS, Lim C and Ferguson LR: In situ and in vitro antioxidant activity of sweetpotato anthocyanins. *J Agric Food Chem* 52: 1511-1513, 2004.

21. Konczak-Islam I, Yoshimoto M, Hou DX, Terahara N and Yamakawa O: Potential chemopreventive properties of anthocyanin-rich aqueous extracts from in vitro produced tissue of sweet potato (*Ipomoea batatas* L.). *J Agr Food Chem* 51: 5916-5922, 2003.
22. de Pascual-Teresa S, Moreno DA and García-Viguera C: Flavanols and anthocyanins in cardiovascular health: A review of current evidence. *Int J Mol Sci* 11: 1679-1703, 2010.
23. Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T and Namba M: Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor- α and angiotensin II. *Circulation* 98: 794-799, 1998.
24. Li HL, Wang AB, Huang Y, Liu DP, Liu G, Zhang CN, Wei C, Liu YQ, Hui RT and Liang CC: Isorhapontigenin, a new resveratrol analog, attenuates cardiac hypertrophy via blocking signaling transduction pathways. *Free Radic Biol Med* 38: 243-257, 2005.
25. Li HL, Huang Y, Zhang CN, Liu G, Wei YS, Wang AB, Liu YQ, Hui RT, Wei C, Williams GM, *et al*: Epigallocatechin-3 gallate inhibits cardiac hypertrophy through blocking reactive oxidative species-dependent and -independent signal pathways. *Free Radic Biol Med* 40: 1756-1775, 2006.
26. Wu TY, Tsai CC, Hwang YT and Chiu TH: Effect of antioxidant activity and functional properties of Chingshey purple sweet potato fermented milk by *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *lactis*, and *L. gasseri* strains. *J Food Sci* 77: M2-M8, 2012.
27. Deschamps AM and Spinale FG: Pathways of matrix metalloproteinase induction in heart failure: bioactive molecules and transcriptional regulation. *Cardiovasc Res* 69: 666-676, 2006.
28. Kang PM and Izumo S: Apoptosis in heart: basic mechanisms and implications in cardiovascular diseases. *Trends Mol Med* 9: 177-182, 2003.
29. Heineke J and Molkenin JD: Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 7: 589-600, 2006.
30. Liu CF, Tung YT, Wu CL, Lee BH, Hsu WH and Pan TM: Antihypertensive effects of *Lactobacillus*-fermented milk orally administered to spontaneously hypertensive rats. *J Agric Food Chem* 59: 4537-4543, 2011.
31. Nishikimi T, Maeda N and Matsuoka H: The role of natriuretic peptides in cardioprotection. *Cardiovasc Res* 69: 318-328, 2006.
32. Chu CH, Tzang BS, Chen LM, Kuo CH, Cheng YC, Chen LY, Tsai FJ, Tsai CH, Kuo WW and Huang CY: IGF-II/mannose-6-phosphate receptor signaling induced cell hypertrophy and atrial natriuretic peptide/BNP expression via G α q interaction and protein kinase C- α /CaMKII activation in H9c2 cardiomyoblast cells. *J Endocrinol* 197: 381-390, 2008.
33. Huang CY, Hao LY and Buetow DE: Hypertrophy of cultured adult rat ventricular cardiomyocytes induced by antibodies against the insulin-like growth factor (IGF)-I or the IGF-I receptor is IGF-II-dependent. *Mol Cell Biochem* 233: 65-72, 2002.
34. Okamura H, Aramburu J and Garcia-Rodriguez C, *et al*: Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* 6: 539-550, 2000.
35. Lim HW and Molkenin JD: Calcineurin and human heart failure. *Nat Med* 5: 246-247, 1999.
36. Kanda T and Takahashi T: Interleukin-6 and cardiovascular diseases. *Jpn Heart J* 45: 183-193, 2004.
37. Meléndez GC, McLarty JL, Levick SP, Du Y, Janicki JS and Brower GL: Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats. *Hypertension* 56: 225-231, 2010.
38. Hirano T: Interleukin 6 and its receptor: ten years later. *Int Rev Immunol* 16: 249-284, 1998.
39. Hirota H, Yoshida K, Kishimoto T and Taga T: Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice. *Proc Natl Acad Sci USA* 92: 4862-4866, 1995.
40. Nicol RL, Frey N, Pearson G, Cobb M, Richardson J and Olson EN: Activated MEK5 induces serial assembly of sarcomeres and eccentric cardiac hypertrophy. *EMBO J* 20: 2757-2767, 2001.
41. Cameron SJ, Itoh S, Baines CP, Zhang C, Ohta S, Che W, Glassman M, Lee JD, Yan C, Yang J and Abe J: Activation of big MAP kinase 1 (BMK1/ERK5) inhibits cardiac injury after myocardial ischemia and reperfusion. *FEBS Lett* 566: 255-260, 2004.
42. Kimura M, Hayakawa K and Sansawa H: Involvement of γ -aminobutyric acid (GABA) B receptors in the hypotensive effect of systemically administered GABA in spontaneously hypertensive rats. *Jpn J Pharmacol* 89: 388-394, 2002.
43. Lin MY and Yen CL: Antioxidative ability of lactic acid bacteria. *J Agric Food Chem* 47: 1460-1466, 1999.