Probiotic-fermented purple sweet potato yogurt activates compensatory IGF-IR/PI3K/Akt survival pathways and attenuates cardiac apoptosis in the hearts of spontaneously hypertensive rats

PEI-PEI LIN[1,9,11], YOU-MIIN HSIEH[2], WEI-WEN KUO[3], YUEH-MIN LIN[4], YU-LAN YEH[4], CHIEN-CHUNG LIN[5], FUU-JEN TSAI[6,7], CHANG-HAI TSAI[8], CHIH-YANG HUANG[8-10] and CHENG-CHIH TSAI[11]

[1]Graduate Institute of Clinical Medical Science, China Medical University; [2]Department of Food and Nutrition, Providence University; [3]Department of Biological Science and Technology, China Medical University, Taichung; [4]Department of Pathology, Changhua Christian Hospital, Changhua; [5]Department of Orthopaedics, Armed Forces General Hospital; [6]Graduate Institute of Chinese Medical Science and [7]Department of Pediatrics, Medical Research and Medical Genetics, China Medical University; Departments of [8]Healthcare Administration and [9]Health and Nutrition Biotechnology, Asia University; [10]Graduate Institute of Basic Medical Science, China Medical University; [11]Department of Food Science and Technology, HungKuang University, Taichung, Taiwan, R.O.C.

Received July 9, 2013; Accepted September 30, 2013

DOI: 10.3892/ijmm.2013.1524

Abstract. Apoptosis is recognized as a predictor of adverse outcomes in subjects with cardiac diseases. The aim of this study was to explore the effects of probiotic-fermented purple sweet potato yogurt (PSPY) with high γ-aminobutyric acid (GABA) content on cardiac apoptosis in spontaneously hypertensive rat (SHR) hearts. The rats were orally adminsitered with 2 different concentrations of PSPY (10 and 100%) or captopril, 15.6 mg/kg, body weight (BW)/day. The control group was administered distilled water. DAPI and TUNEL staining were used to detect the numbers of apoptotic cells. A decrease in the number of TUNEL-positive cardiac myocytes was observed in the SHR-PSPY (10 and 100%) groups. In addition, the levels of key components of the Fas receptor- and mitochondrial-dependent apoptotic pathways were determined by western blot analysis. The results revealed that the levels of the key components of the Fas receptor- and mitochondrial-dependent apoptotic pathway were significantly decreased in the SHR-captopril, and 10 and 100% PSPY groups. Additionally, the levels of phosphorylated insulin-like growth factor-I receptor (p-IGF-IR) were increased in SHR hearts from the SHR-control group; however, no recovery in the levels of downstream signaling components was observed. In addition, the levels of components of the compensatory IGF-IR-dependent survival pathway (p-PI3K and p-Akt) were all highly enhanced in the left ventricles in the hearts form the SHR-10 and 100% PSPY groups. Therefore, the oral administration of PSPY may attenuate cardiomyocyte apoptosis in SHR hearts by activating IGF-IR-dependent survival signaling pathways.

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: apoptosis, lactic acid bacteria, hypertension, fermented yogurt, insulin-like growth factor-I receptor, survival pathway

Introduction

Hypertension is the most common risk factor for congestive heart failure (1), which can induce apoptotic changes in heart function and peripheral vascular resistance (2). Evidence indicates higher levels of activated cardiomyocyte apoptosis and cardiac apoptosis in hypertensive models (3). Since the side-effects of western drugs in the treatment of cardiac diseases cannot be avoided, the investigation of natural products or dietary supplements which can protect cardiac abnormalities and injuries is essential (4).

Apoptosis is a recognized mechanism for the elimination of redundant cells, although it may also inhibit cell proliferation. In fact, it has been suggested that apoptosis plays a critical role in the pathogenesis of cardiac disorders (3,5,6). An increase in the levels of the pro-apoptotic protein, angiotensin II (Ang II), has been observed in hypertension, coronary artery disease, left ventricular hypertrophy and heart failure and may func-

tion as a regulator of apoptosis in cardiac tissues (7,8). Both Fas-dependent and mitochondrial-dependent apoptotic pathways are considered as major pathways which directly cause cardiac apoptosis (9,10).

The 'extrinsic' Fas-dependent apoptotic pathway is initiated by binding the Fas ligand to the Fas death receptor or by binding tumor necrosis factor (TNF)-α to TNF receptor 1, which results in the clustering of receptors, initiating an extrinsic pathway (11). A previous study reported that cardiac Fas receptor-dependent apoptotic pathways were activated in obese rat hearts, which is the one of the possible mechanisms behind cardiac abnormalities in obesity (12). The 'intrinsic' mitochondrial-dependent apoptotic pathway is mediated by internal factors, particularly in the mitochondria, the main site of action for apoptosis-regulating proteins, exemplified by the Bcl-2 family, such as Bax, Bad, t-Bid and Bak (11). Pro-apoptotic and anti-apoptotic Bcl-2 family members can homodimerize or heterodimerize to each other and appear to interact with and neutralize each other, so that the relative balance of these effectors strongly influences the release of cytochrome *c*. When cytochrome *c* is released from the mitochondria into the cytosol, it is responsible for activating caspase-9, which further activates caspase-3 and executes the apoptotic program (13). In a recent study, we observed cardiac mitochondrial-dependent but not Fas-dependent apoptosis in hamsters fed a hyper-cholesterol diet, and demonstrated the cardioprotective effects induced by the activation of the insulin-like growth factor-I receptor (IGF-IR) survival pathway by treatment with Li-Fu formula (4).

Previous studies have indicated that insulin-like growth factor (IGF)-1 signaling contributes to the modulation of survival responses in cardiomyocytes and that low IGF-I levels are associated with a high risk of myocardial infarction and heart failure (11,14). IGF-1 is the survival factor through which IGF-1R activates the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) pathway, thus preventing myocyte apoptosis (15). In particular, activated PI3K enhances the levels of phosphorylated Akt (p-Akt) (16), which in turn regulates the activity of phosphorylated-Bad (p-Bad) and Bcl-2 to prevent the apoptosis of cardiomyocytes (11).

The consumption of γ-aminobutyric acid (GABA)-enriched fermented milk has been reported to depress elevated blood pressure in spontaneously hypertensive rats (SHRs) and mildly hypertensive humans (17-19). In a previous study, we demonstrated that purple sweet potato (PSP) [*Ipomoea batatas* (L.) Lam.] contains high levels of anthocyanin and can easily grow in subtropical areas through fermentation by 3 lactic acid bacteria (LAB), with high levels of GABA (20). However, the protective effects of probiotic-fermented milk and its derivatives, such as GABA on cardiac apoptosis have not been reported. This study was therefore undertaken to examine the potential benefits of GABA-enriched PSP yogurt (PSPY) on the cardiac apoptotic pathways in SHR hearts. We demonstrate that PSPY attenuates cardiac apoptosis by activating the PI3K/AKT survival signaling pathway.

Materials and methods

Bacterial strains and growth conditions. In this study, the 3 experimental LAB strains used were purchased from the Food Industry Research and Development Institute of the Biological Resources Conservation and Research Center: *Lactobacillus acidophilus* BCRC 14065; *L. delbrueckii* subsp. *lactis* BCRC 12256; and *L. gasseri* BCRC 14619. The stock culture was maintained at -80˚C in 20% glycerol prior to use. The bacteria were propagated twice in Lactobacilli MRS broth (Difco, Sparks, MD, USA) containing 0.05% L-cysteine overnight at 37˚C before the experimental procedures.

Preparation of PSPY. PSP was acquired from the Taiwan Agricultural Research Institute and stored at 4˚C after being cleaned with tap water. To prepare the PSPY, the potatoes were first peeled, cut into 1-cm-thick slices and steamed at 100˚C for 20 min. The cooked spuds were then homogenized and then 0.05% α-amylase, 10% skimmed milk powder, 0.05% protease and 3% whey protein were added to the mixture before pasteurization (121˚C, 15 min). The 3 different LAB strains were inoculated at a concentration of 10^9 CFU/ml 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 a refrigerator for use in subsequent experiments.

Animals and experimental groups. Twenty-two male SHRs and 12 male Wistar Kyoto (WKY) rats were purchased from BioLASCO Taiwan Co., Ltd, Taipei, Taiwan. The animals (aged 6 weeks) were housed individually in a temperature (20±2˚C)- and humidity (55±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. The rats were allowed access to food [chow pellets (MF-18; Oriental Yeast Co. Ltd., Tokyo, Japan)] and water *ad libitum*. An acclimatization period of 1 week after delivery by the supplier was allowed before the SHRs were randomly divided into the following 4 groups: i) the SHR control group (rats administered 2.5 ml distilled water); ii) the anti-hypertensive captopril group [rats administered captopril, 15.6 mg/kg, body weight (BW)/day]; iii) the 10% PSPY group (rats administered a 10-fold dilution of PSPY, 1.068 μg GABA/2.5 ml PSPY); and iv) the 100% PSPY group (rats administered undiluted PSPY, 10.68 μg GABA/2.5 ml PSPY). Experimental feeding concentrations of PSPY were calculated on the basis of GABA concentrations by the high-performance liquid chromatography (HPLC) method. Captopril and PSPY were orally administered daily to the rats until the rats were sacrificed on the 8th week of the experimental period. WKY rats were used as the negative control. 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.

4',6-diamidine-2-phenylindole dihydrochloride (DAPI) staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL). After the hearts were excised, the hearts were soaked in formalin, dehydrated through graded alcohols and embedded in paraffin wax. From the heart tissues, the 3-μm-thick paraffin sections were cut from the paraffin-embedded tissue blocks. The sections were deparaffinized by immersing them in xylene (5 min, thrice), rehydrated and incubated in phosphate-buffered saline (PBS), pH 7.4, with

3% H_2O_2 to inactivate endogenous peroxidase. The sections were then incubated with proteinase K (20 μg/ml) for 30 min, washed in 0.1 M PBS, and incubated with TUNEL reaction mixture for 60 min at 37˚C using an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA). After washing with PBS twice, the sections were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 5 min to detect cell nuclei by UV light microscopic observations (blue). TUNEL-positive nuclei (fragmented DNA) fluoresced bright green at 450-500 nm, whereas DAPI-positive nuclei (intact DNA) fluoresced blue at 360 nm. The mean numbers of TUNEL-positive and DAPI-labeled cells were counted for at least 3 separate fields from 2 slides excised from the rat hearts in each group. All counts were performed by at least 2 independent individuals in a blinded manner.

Tissue extraction. The left ventricle was cut into 8 sections. One section from the left ventricle was minced with scissors; then lysis buffer (20 mM Tris, 2.0 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, pH 7.4), a proteinase inhibitor cocktail tablet and phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) were added at a concentration of 100 mg tissue/ml buffer and the mixture was homogenized on ice using a Model PT 10/35 Polytron homogenizer for 2 cycles of 10 sec each. The homogenate was then 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 content. The protein content of the left ventricle extracts was determined using the Bradford protein assay using the protein-dye kit (Bio-Rad, Richmond, CA, USA). Commercially available bovine serum albumin (Sigma-Aldrich) was used as a standard. Changes in absorption were monitored at 595 nm.

Electrophoresis and western blot analysis. The left ventricle extracts 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]. Subseqently, the electrophoresed proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (0.45 μm pore size) (Millipore, Bedford, MA, USA) using a Bio-Rad Scientific Instruments Transphor Unit at 100 V with transfer buffer for 3 h. The PVDF membranes were incubated at room temperature for 1 h in 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 against Akt (BD Pharmingen, San Jose, CA, USA), p-Akt (Cell Signaling Technology, Inc., Danvers, MA, USA) and polyclonal antibodies against Fas, Bid, t-Bid, Bcl-xL, PI3K, phoshorylated PI3K (p-PI3K), Bak, Bax, caspase-3, α-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), cleaved caspase-9, p-Bad (Cell Signaling Technology, Inc.) and IGF-IR and phosphorylated-IGF-IR (p-IGF-IR) (Abcam, Taipei, Taiwan) 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 secondary 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 by using enhanced chemiluminescence (ECL) Western Blotting Luminal Reagent (Santa Cruz Biotechnology, Inc.) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Fujifilm, Tokyo, Japan). 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 $MgCl_2$ 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 an internal control.

Statistical analysis. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data were compared between the animal groups 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. Significant differences are indicated with symbols, as shown in the tables and figures.

Results

TUNEL-positive apoptotic cells in cardiac tissues. We wished to determine the effects of PSPY on hypertension-induced apoptosis in cardiac cells. We examined the TUNEL-positive cardiac cells in the excised hearts from the WKY rats, and from the SHRs in the control, captopril and PSPY (both concentrations, 10 and 100%) groups by TUNEL assay. Following TUNEL staining, an increased number of TUNEL-positive cardiac cells was observed in the left ventricle in the SHR-control group compared with the WKY group (Fig. 1A). Notably, a significantly reduced number of TUNEL-positive cardiac cells was found in the left ventricle of the SHR hearts in the captopril, and 10 and 100% PSPY groups compared with the SHR-control group (Fig. 1A). The percentage of TUNEL-positive cardiac cells was calculated and the quantified results are shown in Fig. 1B.

Changes in the levels of Fas death receptor-related components in the hearts of SHRs fed different concentrations of PSPY. We examined the variations in the levels of Fas death receptor-associated proteins in the hearts of SHRs administered captopril and various concentrations of PSPY by western blot analysis (Fig. 2). The protein products of Fas ligands and Fas extracted from the left ventricles of the excised hearts in the SHR-control group were significantly increased compared with the WKY group (Fig. 2). By contrast, significantly decreased levels of protein products of Fas ligands were detected in the SHR hearts from the captopril, and 10 and 100% PSPY groups compared with the SHR-control group ($P<0.05$) (Fig. 2B). The levels of the protein products of Fas were significantly decreased in the SHR-100% PSPY group, compared with the SHR-control group ($P<0.05$) (Fig. 2C). The levels of protein products of Fas were slightly decreased in the SHR hearts

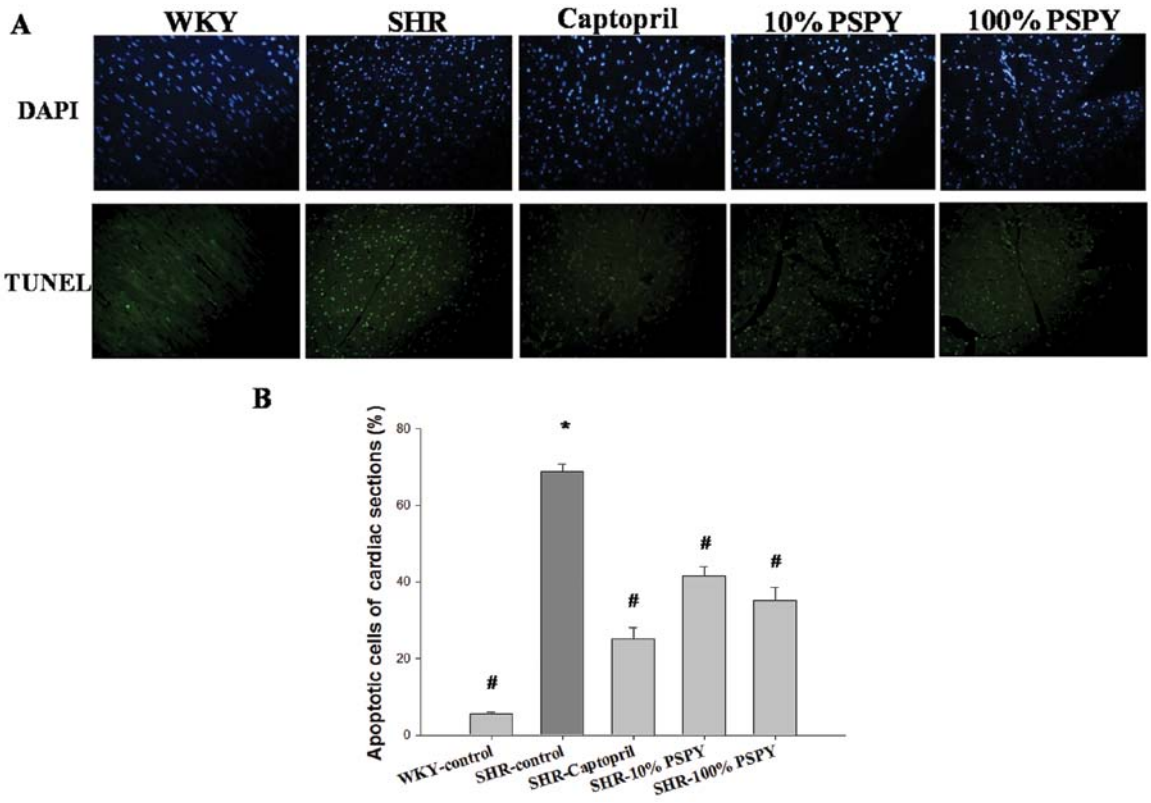

Figure 1. Representative stained apoptotic cells of cardiac sections from left ventricles in Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). SHRs were treated captopril (SHR-captopril), 10% probiotic-fermented purple sweet potato yogurt (PSPY) and 100% PSPY (SHR-10% PSPY and SHR-100% PSPY). (A) DAPI staining (upper panels, blue spots) and TUNEL staining (lower panels, green spots) was performed to detect apoptosis; magnification, x400. (B) The percentages of apoptotic cells were calculated. Bars present the percentage of TUNEL-positive cells relative to that of total cells (3 mice x10 scope field count in each group) Values are the means ± SD. *P<0.05 compared with the WKY group. #P<0.05, compared with the SHR-control group.

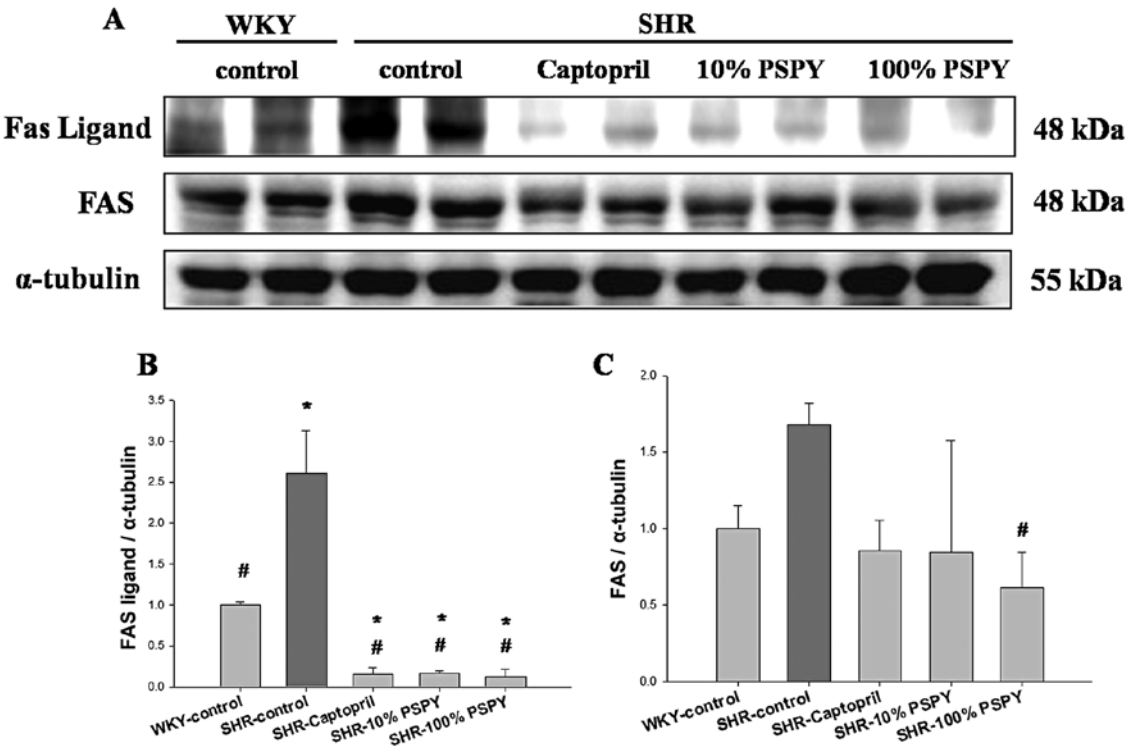

Figure 2. Effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the presence of Fas ligand and Fas proteins. (A) Protien levels of Fas ligand and Fas in the left ventricles of excised hearts from rats in the Wistar Kyoto (WKY), spontaneously hypertensive rat (SHR), SHR-captopril, SHR-10% PSPY and SHR-100% PSPY groups, as measured by western blot analysis. (B and C) Relative protein quantification of Fas ligand and Fas on the basis of α-tubulin; values are the means ± SD (n=3 in each group). *P<0.05 compared with the WKY group. #P<0.05 compared with the SHR-control group.

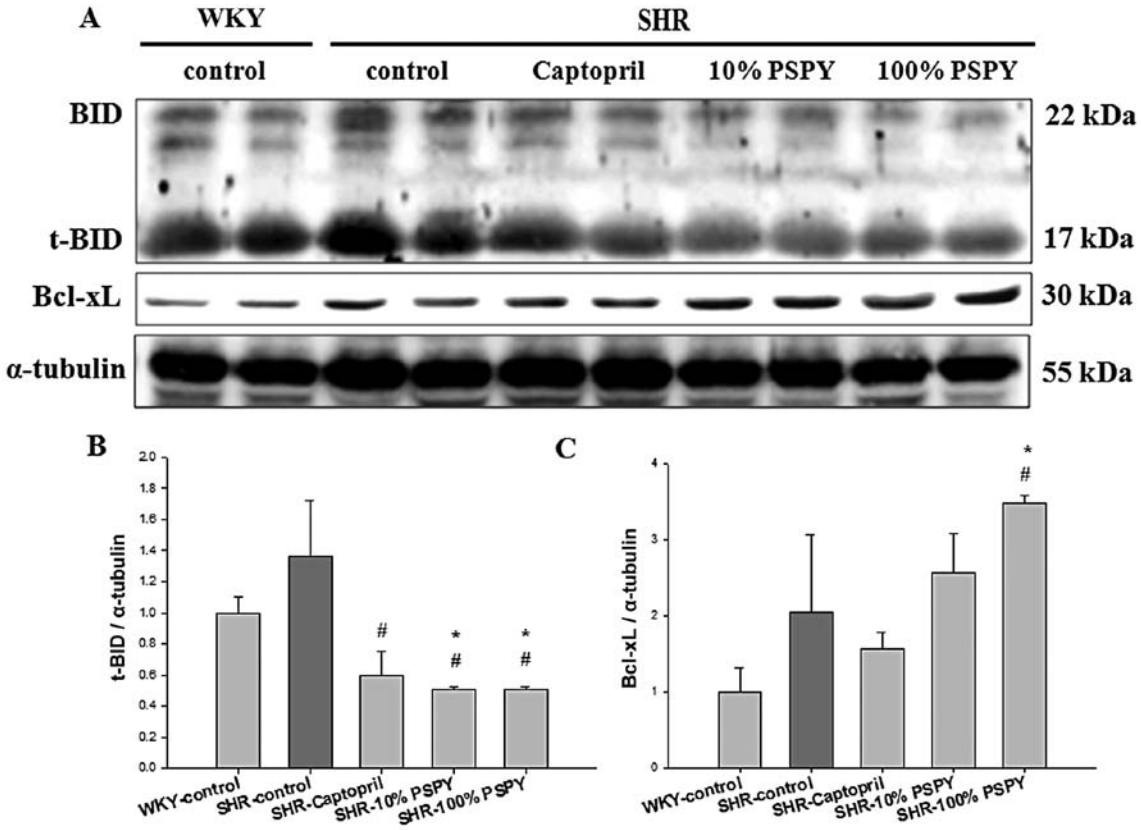

Figure 3. Effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the presence of BID, t-BID and Bcl-xL proteins. (A) The protein leels of BID, t-BID and Bcl-xL in the left ventricles of excised hearts from rats in the Wistar Kyoto (WKY), spontaneously hypertensive rat (SHR), SHR-captopril, SHR-10% PSPY and SHR-100% PSPY groups, as measured by western blot analysis. (B and C) Relative protein quantification of t-BID and Bcl-xL on the basis of α-tubulin; values are the means ± 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 captopril and 10% PSPY groups, and these levels did not differ significantly from those in the SHR-control group (Fig. 2C). The ratios of the protein products of Fas ligands and Fas relative to α-tubulin were calculated and are shown in Figs. 2B and C.

Changes in the levels of mitochondrial-dependent apoptotic components in the hearts of SHRs fed different concentrations of PSPY. We examined the variations in the levels of mitochondrial-dependent apoptotic components in the cardiac tissues of SHRs fed PSPY. The protein levels of Bcl-2 family members (t-Bid, Bcl-xL, Bak, Bax and p-Bad) were examined by western blot analysis (Figs. 3 and 4). Significantly decreased levels of the protein products of t-Bid were detected in the left ventricles of excised SHR hearts from the captopril, and 10 and 100% PSPY groups, compared with the SHR-control group (P<0.05); however, the SHR-control group showed no significant difference to the WKY group (Fig. 3B). In addition, the levels of anti-apoptotic proteins (Bcl-xL) were significantly increased in the SHR hearts in the 100% group compared with the WKY and SHR-control groups (Fig. 3C). However, the levels of mitochondrial related pro-apoptotic proteins (Bak and Bax) were significantly increased in the SHR-control group, compared with the WKY group (P<0.05) (Fig. 4), whereas significantly decreased levels of Bak and Bax protein were detected in the SHR hearts from the 100% PSPY group, compared with the SHR-control group (Fig. 4B and C). Moreover, the protein levels of activated caspase-9 did not differ significantly among the groups (Fig. 5B). By contrast, the levels of activated caspase-3 were significantly increased in the SHR-control group compared with the WKY group (P<0.05) (Fig. 5C), and were significantly decreased in the SHR hearts from the 10 and 100% PSPY groups compared with the SHR-control group (Fig. 5C).

Change in the levels of cardiac survival signaling components in the hearts of SHRs fed different concentrations of PSPY. In order to determine the effects of PSPY on cardiac IGF-IR-dependent survival pathways, we examined the protein levels of IGF-IR, p-IGF-IR and IGF-IR signaling components, including PI3K, p-PI3K, AKT and p-AKT in the rat hearts. The protein levels of IGF-IR were significantly decreased in the SHR hearts from the SHR-control, captopril, and 10 and 100% PSPY groups compared with the WKY group (P<0.05) (Fig. 6). However, the protein levels of p-IGF-1R were significantly increased in the SHR hearts from the SHR-control group compared with the WKY group. The SHR-10 and 100% PSPY groups showed even higher levels of p-IGF-IR than the SHR-control group (P<0.05) (Fig. 6C). Notably, we found significantly increased levels of PI3K, p-PI3K, AKT and

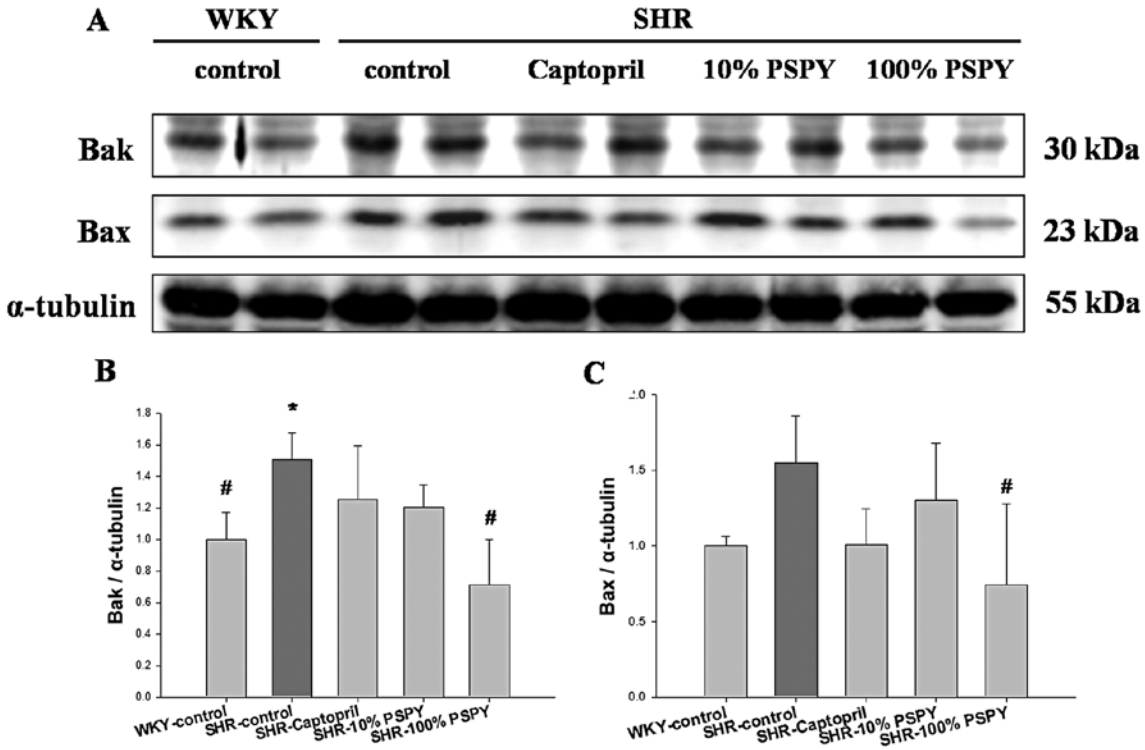

Figure 4. Effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the presence of Bak and Bax proteins. (A) The protein levels of Bak and Bax in the left ventricles of the excised hearts from rats in the Wistar Kyoto (WKY), spontaneously hypertensive rat (SHR), SHR-captopril, SHR-10% PSPY and SHR-100% PSPY groups, as measured by western blot analysis. (B and C) Relative protein quantification of Bak, and Bax on the basis of α-tubulin; values are the means ± SD (n=3 in each group). *P<0.05 compared with the WKY group; #P<0.05 compared with the SHR-control group.

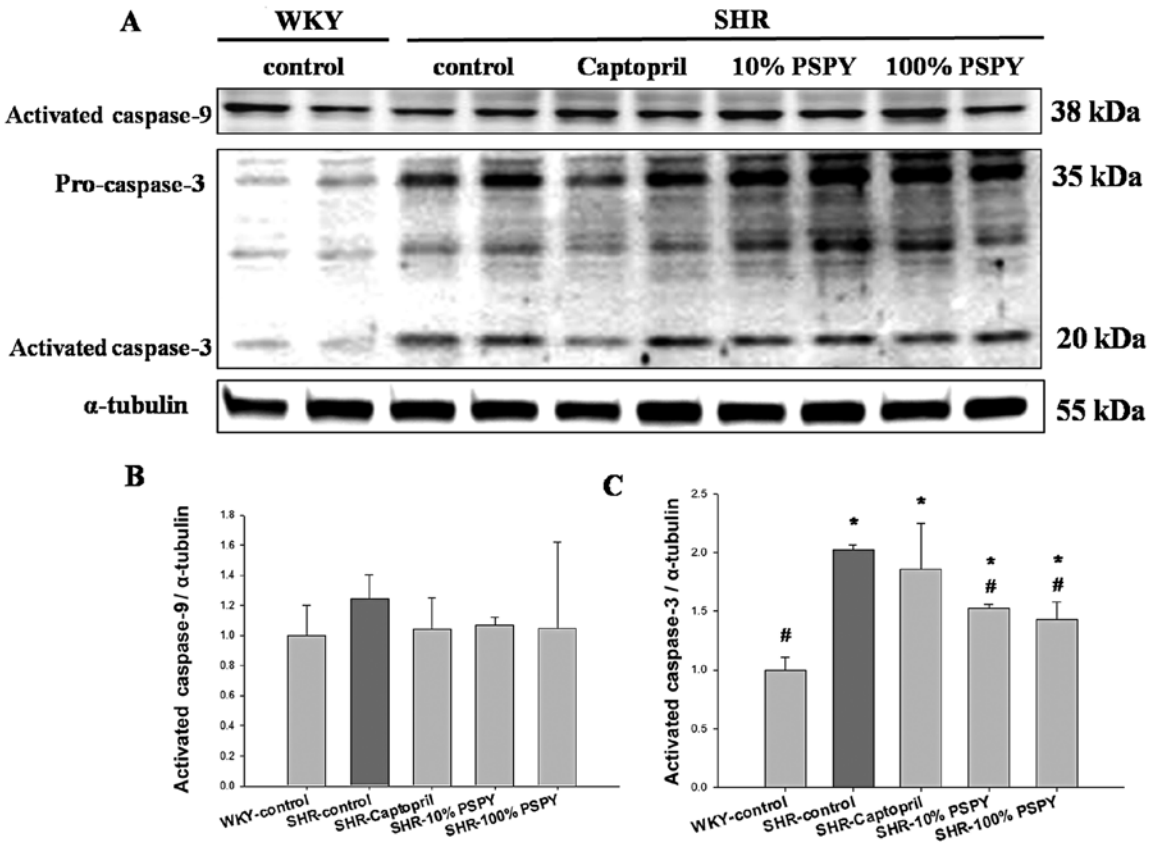

Figure 5. Effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the presence of caspase-9 and caspase-3 proteins. (A) The protein levels of caspase-9 and caspase-3 in the left ventricles of the excised hearts from rats in the Wistar Kyoto (WKY), spontaneously hypertensive rat (SHR), SHR-captopril, SHR-10% PSPY and SHR-100% PSPY groups, as measured by western blot analysis. (B and C) Relative protein quantification of caspase-9 and caspase-3 on the basis of α-tubulin; values are the means ± SD (n=3 in each group). *P<0.05 compared with the WKY group. #P<0.05 compared with the SHR-control group.

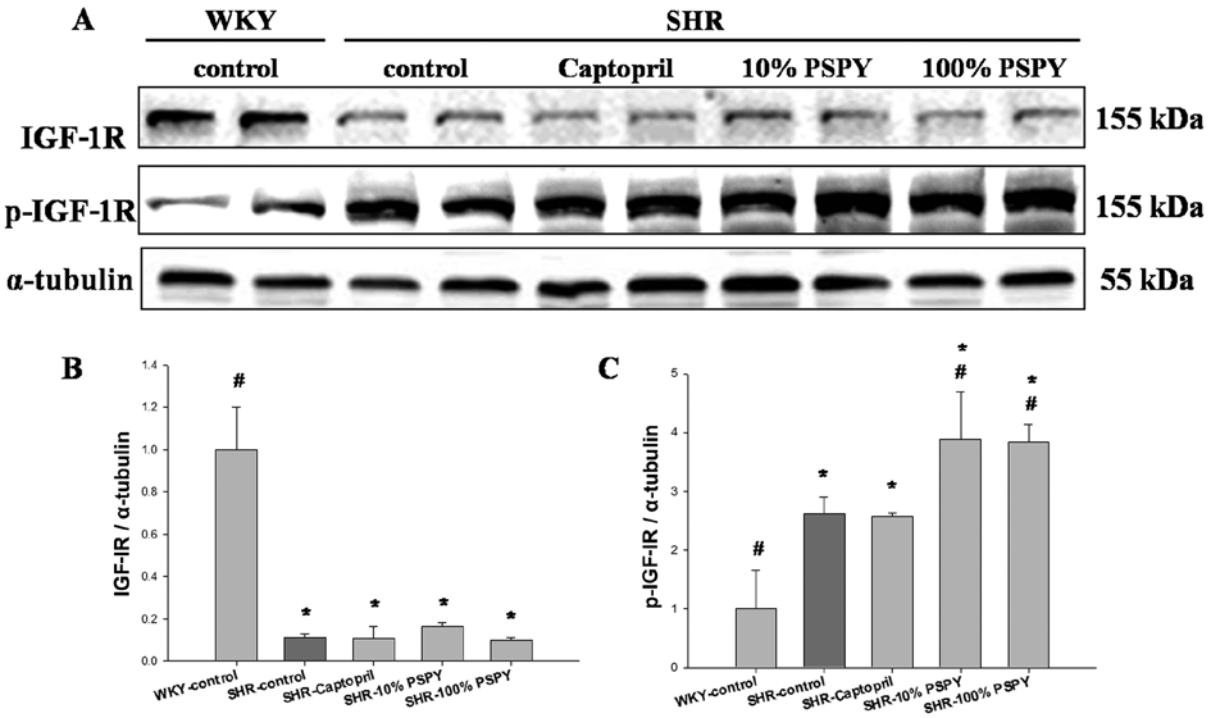

Figure 6. Effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the presence of insulin-like growth factor-I receptor (IGF-IR) and phosphorylated (p)-IGF-IR proteins. (A) The protein levels of IGF-IR and p-IGF-IR in the left ventricles of the excised hearts from rats in the Wistar Kyoto (WKY), spontaneously hypertensive rat (SHR), SHR-captopril, SHR-10% PSPY and SHR-100% PSPY groups, as measured by western blot analysis. (B and C) Relative protein quantification of IGF-IR and p-IGF-IR on the basis of α-tubulin; values are the means ± SD (n=3 in each group). *P<0.05 compared with the WKY group. #P<0.05 compared with the SHR-control group.

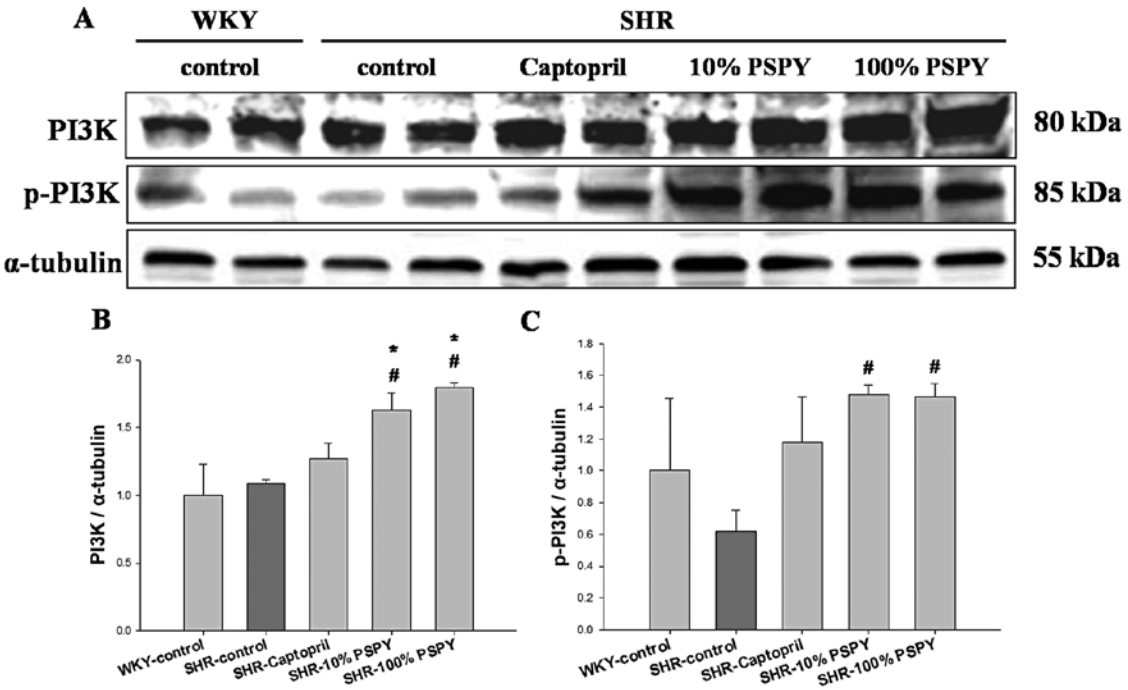

Figure 7. Effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the presence of PI3K and p-PI3K proteins. (A) The protein levels of PI3K and p-PI3K in the left ventricles of excised hearts from the rats in the Wistar Kyoto (WKY), spontaneously hypertensive rat (SHR), SHR-captopril, SHR-10% PSPY and SHR-100% PSPY groups, as measured by western blot analysis. (B andC) Relative protein quantification of PI3K and p-PI3K on the basis of α-tubulin; values are the means ± SD (n=3 in each group). *P<0.05 compared with the WKY group. #P<0.05 compared with the SHR-control group.

p-AKT in the SHR hearts from the 10 and 100% PSPY groups compared with the SHR-control group (P<0.05) (Figs. 7 and 8). By contrast, no significant increase in the levels of cardiac PI3K, p-PI3K and p-AKT was observed in the SHR hearts from the control and captopril groups compared with the WKY group (Figs. 7 and 8).

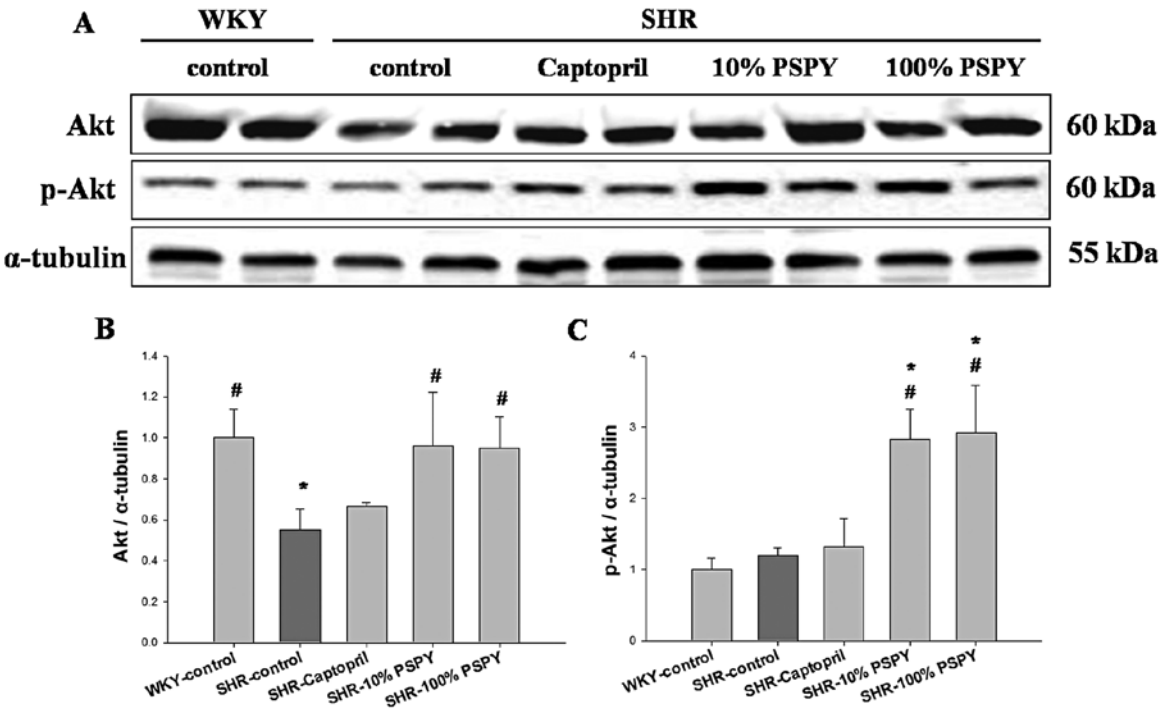

Figure 8. Effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the presence of AKT and p-AKT proteins. (A) The protein levels of AKT and p-AKT in the left ventricles of excised hearts from rats in the Wistar Kyoto (WKY), spontaneously hypertensive rat (SHR), SHR-captopril, SHR-10% PSPY and SHR-100% PSPY groups, as measured by western blot analysis. (B and C) Relative protein quantification of AKT and p-AKT on the basis of α-tubulin; values are the means ± SD (n=3 in each group). *P<0.05 compared with the WKY group. #P<0.05 compared with the SHR-control group.

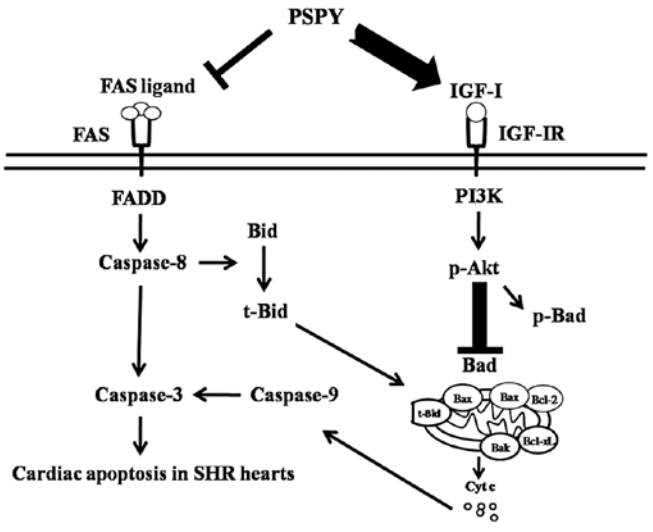

Figure 9. Diagram of the suggested pathways activated in spontaneously hypertensive rats (SHRs) and the effects of probiotic-fermented purple sweet potato yogurt (PSPY). PSPY inhibits the activation of cardiac Fas- and mitochondrial-dependent apoptotic pathways in SHRs. Cardiac Fas- and mitochondrial-dependent apoptotic pathways are activated in SHRs and the supplementation of PSPY activates cardiac IGFI-R/PI3K/Akt survival pathways and anti-apoptotic Bcl-2 family (Bcl-xL)-associated pathways in SHRs.

Discussion

The major findings of the present study can be summarized as follows: i) a higher number of TUNEL-positive apoptotic cells was observed in the SHR-control group than in the WKY group, whereas a significantly reduced number of TUNEL-positive cells was observed in the 10 and 100% PSPY groups. ii) The levels of Fas receptor- and mitochondrial-dependent apoptotic signaling pathway components, such as Fas ligand, Fas, t-BID, Bak, Bax and activated caspase-3 were significantly reduced and those of anti-apoptotic proteins (Bcl-xL and p-Bad) were increased in the left ventricle tissues in the SHR hearts from the 10 and 100% PSPY groups compared with the SHR-control group. iii) A significant decrease in the levels of IGF-IR downstream-associated components was detected in the excised ventricle tissues from the SHR-control group even with the increase in the compensatory activated form (p-IGF-IR). However, the increased levels of p-IGF-IR significantly increased IGF-IR signaling components, such as p-IGF-IR, p-PI3K and p-AKT, as observed in the SHR hearts from rats fed 10 and 100% PSPY. After integrating our current findings into previously proposed apoptotic theories, a hypothesized path diagram was created (Fig. 9), which suggested that cardiac Fas receptor-dependent and mitochondrial-dependent pathways may be activated in hypertensive rats and may be suppressed by the supplementation of PSPY. By contrast, the compensatory IGF-IR pathway was activated, but the levels of the downstream survival components were still decreased in the hypertensive rats; these levels increased by the supplementation of PSPY; PSPY completely restored the survival mechanisms. Our findings demonstrate the novel therapeutic effects of PSPY in hypertensive rat hearts; PSPY can be used to prevent apoptosis and enhance survival. It seemed to be more effective than captopril.

Chronic hypertension is a major risk factor for the development of cardiovascular diseases (1). Hypertension induces pathological cardiac hypertrophy secondary to the pressure

overload, thereby contributing to decreased cardiac function and increased cardiac apoptosis (21,22). In a previous study, we demonstrated that PSPY exerted anti-hypertrophic effects in the hearts of SHRs by interfering with the IGF-II signaling pathway and the IL-6-related-ERK5 pathway (23). In the present study, the results of cardiomyocyte loss, as shown by TUNEL assay are consistent with those of previous studies describing the gradual cardiac decompensation in SHR hearts (24). Similar results have also been reported in a recent study, where the authors observed that the left ventricles of SHR hearts had more TUNEL-positive cardiac cells than those in the WKY group (25).

The balance between cell death and survival is a tightly controlled process, particularly in terminally differentiated cells, such as cardiomyocytes (21). The Fas receptor-dependent apoptotic pathway is mediated by the Fas ligand, the Fas receptor, TNF-α, the TNF receptor, Fas-associating death domain-containing protein (FADD) and the activation of caspase-8 (5,11). In our findings, PSPY significantly prevented the activation of the Fas receptor-dependent apoptotic pathways in SHR hearts, as evidenced by the decrease in the levels of hypertension-upregulated Fas ligand and Fas receptor after the oral administration of 10 and 100% PSPY. To our knowledge, the present study is the first to illustrate that PSPY prevents the activation of cardiac Fas receptor-dependent apoptotic pathways in hypertensive rats.

The mitochondrial-dependent apoptotic pathway is tightly controlled by the Bcl-2 family. Pro-apoptotic and anti-apoptotic members of the Bcl-2 family seem to interact with and neutralize each other, so that the relative balance of these effectors strongly influences cell fate (26). Shifting the balance of Bcl-2 family members toward pro-apoptotic members activates caspase-9, which further activates caspase-3 and executes the apoptotic program (13). In the present study, PSPY prevented the activation of pro-apoptotic members of the Bcl-2 family in SHR hearts, as evidenced by the decrease in the levels of hypertension-upregulated components, t-Bid, Bak and Bax, after the supplementation of PSPY in the 10 and 100% groups. The supplementation of PSPY significantly increased the levels of anti-apoptotic components due to the elevation of Bcl-xL and p-Bad levels, and thus decreased activated caspase-3 levels in the hypertensive rat hearts. Therefore, our results strongly suggest that the oral administration of PSPY in SHRs may prevent the activation of cardiac apoptotic pathways.

The cardiac survival pathway can be mediated by IGF-I-related survival pathways, such as IGF-I, IGF-IR, p-PI3K and p-Akt. Previous studies have indicated that increased Bcl-xL levels in the mitochondria were observed in IGF-I pre-treated rats and that cardiac-specific IGF-I overexpression has an anti-apoptotic function; however, increased apoptosis followed by myocardial infarction was observed in IGF-I-deficient mice (27,28). Consistent with previous findings, our results demonstrated that a significant decrease in the levels of IGF-IR pathway-associated components was detected in the ventricles from the excised SHR hearts. By contrast, the oral administration of 10 and 100% PSPY activated the compensatory cardiac survival pathway in SHR hearts, evidenced by the increased levels of p-IGF-IR, and restored the levels of PI3K, p-PI3K, Akt and p-Akt. Similar results were also reported in a previous study, demonstrating that the IGF-I/PI3K/Akt survival pathway was activated in the SHR-exercise training groups compared with SHR and WKY groups (25). These findings suggest that PSPY attenuates cardiac apoptosis and facilitates the activation of the compensatory IGFI/PI3K/Akt survival pathway.

The 2 concentrations (10 and 100%) of PSPY exerted a significant effect on cardiac apoptosis and the IGF-I survival pathway in our study, although there was no significant concentration-response effect observed between the 10-fold differences in PSPY concentrations. We speculate that the component of PSPY may contain other elements apart from GABA, whose biological function has yet been elucidated, regardless of the original presumption that the cardiac anti-apoptotic effects came from the presence of GABA. Various compounds of plants with antioxidant properties have been shown to exert a number of therapeutic effects in animal models of hypertension, which supports the hypothesis that reactive oxygen species (ROS) are involved in the progression of hypertension (29). In a previous study, we demonstrated that the fermentation procedure could further elevate anthocyanin levels and antioxidative activity significantly in PSPY with multi-strain probiotics (20). Additionally, the supplementation of probiotics may influence host intestinal microflora and subsequently improve cardiovascular functions. Lam *et al* (30) demonstrated that the oral administration of the commercially available probiotic juice (*L. plantarum* 299v) reduced myocardial infarct size in Dahl S rats. Sobol *et al* (31) considered that LAB and their metabolic products in particular may positively affect the calcium signal in cells in the cardiovascular system, which may result in increased contractile activity of blood vessels and cardiac cells. Based on previous findings, we hypothesized that anthocyanidin-enriched purple potato and the probiotics in PSPY may also be the components that potentially contribute to the activation of the PI3K/AKT survival pathway and the attenuation of cardiac apoptosis, and account for the insignificant difference between the 2 PSPY dosages.

In conclusion, hypertension is considered a major risk factor for the development of heart failure. Our current findings indicate that impaired cardiac IGF-I/PI3K/Akt survival and Bcl-2 family anti-apoptotic pathways in hypertensive rats may provide an important mechanism to explain the development of hypertensive heart diseases. Additionally, the supplementation of PSPY in the diet, found to be beneficial by enhancing cardiac survival and activating anti-apoptotic pathways in hypertensive hearts, may be considered as a potential novel therapeutic strategy to prevent the development of apoptosis-related cardiac diseases in hypertension. Further clinical experiments are required to clarify the survival and apoptotic mechanisms responsible for the beneficial effects of PSPY in human hypertensive hearts.

Acknowledgements

This study was supported in part by grants form the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH102-TD-B-111-004), the Taiwan Department of Health Cancer Research Center of Excellence (DOH102-TD-C-111-005) and the National Science Council, Taiwan, R.O.C. (NSC97-2313-B-241-004-MY3).

References

1. Tocci G, Sciarretta S and Volpe M: Development of heart failure in recent hypertension trials. J Hypertens 26: 1477-1486, 2008.
2. Morillas P, de Andrade H, Castillo J, Quiles J, Bertomeu-González V, Cordero A, Tarazón E, Roselló E, Portolés M, Rivera M and Bertomeu-Martínez V: Inflammation and apoptosis in hypertension. Relevance of the extent of target organ damage. Rev Esp Cardiol 65: 819-825, 2012.
3. 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.
4. Kuo WW, Hsu TC, Chain MH, Lai CH, Wang WH, Tsai FJ, Tsai CH, Wu CH, Huang CY and Tzang BS: Attenuated cardiac mitochondrial-dependent apoptotic effects by li-fu formula in hamsters fed with a hypercholesterol diet. Evid Based Complement Alternat Med 2011: 530345, 2011.
5. Haunstetter A and Izumo S: Apoptosis: basic mechanisms and implications for cardiovascular disease. Circ Res 82: 1111-1129, 1998.
6. Narula J, Haider N, Arbustini E and Chandrashekhar Y: Mechanisms of disease: apoptosis in heart failure - seeing hope in death. Nat Clin Pract Cardiovasc Med 3: 681-688, 2006.
7. Harrison DG, Cai H, Landmesser U and Griendling KK: Interactions of angiotensin II with NAD(P)H oxidase, oxidant stress and cardiovascular disease. J Renin Angiotensin Aldosterone Syst. 4: 51-61, 2003.
8. Filippatos G, Tilak M, Pinillos H and Uhal BD: Regulation of apoptosis by angiotensin II in the heart and lungs (Review). Int J Mol Med 7: 273-280, 2001.
9. Fujio Y, Nguyen T, Wencker D, Kitsis RN and Walsh K: Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. Circulation 101: 660-667, 2000.
10. Athanasiou A, Clarke AB, Turner AE, Kumaran NM, Vakilpour S, Smith PA, Bagiokou D, Bradshaw TD, Westwell AD, Fang L, Lobo DN, Constantinescu CS, Calabrese V, Loesch A, Alexander SP, Clothier RH, Kendall DA and Bates TE: Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. Biochem Biophys Res Commun 364: 131-137, 2007.
11. Bishopric NH, Andreka P, Slepak T and Webster KA: Molecular mechanisms of apoptosis in the cardiac myocyte. Curr Opin Pharmacol 1: 141-150, 2001.
12. Lee SD, Tzang BS, Kuo WW, Lin YM, Yang AL, Chen SH, Tsai FJ, Wu FL, Lu MC and Huang CY: Cardiac fas receptor-dependent apoptotic pathway in obese Zucker rats. Obesity (Silver Spring) 15: 2407-2415, 2007.
13. Brown GC and Borutaite V: Nitric oxide, cytochrome c and mitochondria. Nitric oxide, cytochrome c and mitochondria. Biochem Soc Symp 66: 17-25, 1999.
14. Ren J, Samson WK and Sowers JR: Insulin-like growth factor I as a cardiac hormone: physiological and pathophysiological implications in heart disease. J Mol Cell Cardiol 31: 2049-2061, 1999.
15. Vincent AM and Feldman EL: Control of cell survival by IGF signaling pathways. Growth Horm IGF Res 12: 193-197, 2002.
16. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW and Liao JK: Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407: 538-541, 2000.
17. 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.
18. 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.
19. 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.
20. Wu TY, Tsai CC, Hwang YT and Chiu TH: Effect of anti-oxidant 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.
21. Fortuno MA, Ravassa S, Fortuno A, Zalba G and Diez J: Cardiomyocyte apoptotic cell death in arterial hypertension: mechanisms and potential management. Hypertension 38: 1406-1412, 2001.
22. Richey PA and Brown SP: Pathological versus physiological left ventricular hypertrophy: a review. J Sports Sci 16: 129-141, 1998.
23. Lin PP, Hsieh YM, Kuo WW, Lin CC, Tsai FJ, Tsai CH, Huang CY and Tsai CC: Inhibition of cardiac hypertrophy by probiotic-fermented purple sweet potato yogurt in spontaneously hypertensive rat hearts. Int J Mol Med 30: 1365-1375, 2012.
24. Díez J, Fortuño MA and Ravassa S: Apoptosis in hypertensive heart disease. Curr Opin Cardiol 13: 317-325, 1998.
25. Huang CY, Yang AL, Lin YM, Wu FN, Lin JA, Chan YS, Tsai FJ, Tsai CH, Kuo CH and Lee SD: Anti-apoptotic and pro-survival effects of exercise training on hypertensive hearts. J Appl Physiol 112: 883-891, 2012.
26. McGowan BS, Ciccimaro EF, Chan TO and Feldman AM: The balance between pro-apoptotic and anti-apoptotic pathways in the failing myocardium. Cardiovasc Toxicol 3: 191-206, 2003.
27. Torella D, Rota M, Nurzynska D, Musso E, Monsen A, Shiraishi I, Zias E, Walsh K, Rosenzweig A, Sussman MA, Urbanek K, Nadal-Ginard B, Kajstura J, Anversa P and Leri A: Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 94: 514-524, 2004.
28. Palmen M, Daemen MJ, Bronsaer R, Dassen WR, Zandbergen HR, Kockx M, Smits JF, van der Zee R and Doevendans PA: Cardiac remodeling after myocardial infarction is impaired in IGF-1 deficient mice. Circ Res 50: 516-524, 2001.
29. Wong CM, Bansal G, Pavlickova L, Marcocci L and Suzuki YJ: Reactive oxygen species and antioxidants in pulmonary hypertension. Antioxid Redox Signal 18: 1789-1796, 2012.
30. Lam V, Su J, Koprowski S, Hsu A, Tweddell JS, Rafiee P, Gross GJ, Salzman N H and Baker JE: Intestinal microbiota determine severity of myocardial infarction in rats. FASEB J 26: 1727-1735, 2012.
31. Sobol KV, Belostotskaya GB and Nesterov VP: The effect of probiotics and their metabolic products on cardiovascular system cells in vitro. Dokl Biol Sci 436: 9-12, 2011.