Suppression of TLR-4-Related Inflammatory Pathway and Anti-Fibrosis Effects of Probiotic-Fermented Purple Sweet Potato Yogurt in Hearts of Spontaneously Hypertensive Rats

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Abstract

Inflammation plays an important role in triggering fibrosis of cardiovascular disease and hypertension. Gamma-aminobutyric acid (GABA) has hypotensive effect; GABA concentration could be enhanced in milk fermented with lactic acid bacteria (LAB). This study evaluated the effect of probiotic-fermented purple sweet potato yogurt (PSPY) on the toll-like receptor 4 (TLR-4)-related inflammatory components, and on fibrosis in the heart of spontaneously hypertensive rat (SHR). TLR4-related pathway and fibrosis-associated proteins TGFβ and FGF2 were significantly increased in SHR hearts, but were highly suppressed in 10% PSPY-fed rats. Microscopic examination with Masson trichrome staining of left ventricle further demonstrated that 10% and 100% PSPY both significantly reduced interstitial fibrosis in SHR hearts. These findings indicated that oral administration of 10% probiotic-fermented PSPY was strong enough to lower cardiac fibrosis in SHR rats through the suppression of TLR-4-related inflammatory pathway. Therefore, PSPY may be included in diets to help prevent cardiac fibrosis in patients with hypertension.

Key Words: hypertension, inflammation, fibrosis, lactic acid bacteria, TLR-4, purple sweet potato
Introduction

Cardiovascular fibrosis is the most common consequence of hypertensive disease and contributes to the development of cardiovascular dysfunction (8). Fibrosis is thought to develop as a result of a tissue repair process associated with excessive chronic inflammation (39), the common major process for the development of cardiovascular disease which could be controlled for therapeutic purposes (22).

Nuclear factor-κB (NFκB) is an ubiquitous eukaryotic proinflammatory transcription factor which is noncovalently bound to cellular inhibitor of κB (IκB) (15). Various stimuli, such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS), can activate IκB kinases that phosphorylate IκB, leading to subsequent IκB ubiquitination and proteasomal degradation. NFκB released from the IκB/NFκB complex then translocates into the nucleus, where it initiates the expression of inflammation genes and further participates in the tissue repair and remodeling processes (9, 39). This tightly-controlled signaling pathway is implicated in cardiac pathologies and is also applied in the development of new therapeutic strategies to manage heart diseases.

TNF-α is a multifunctional pro-inflammatory cytokine and early mediator of the acute-phase response that is caused by infection or tissues damage. TNF-α is responsible for induction of the mentioned fever and muscle catabolism, and they activate white blood cell precursors in the bone marrow, growth of inflammatory tissue fibroblasts and macrophages to influence the specific immune response of the stressed organism against foreign antigens and invading microorganisms. A high TNF-α level in centenarians is, thus, associated with a low ankle-brachial arterial pressure index, demonstrating peripheral atherosclerosis and heart diseases (3). In LPS-stimulated myocardial, increasing amounts of TNF-α are locally synthesized by cardiac myocytes in chronic heart failure, cardiac sudden death, viral myocarditis and ischemia (35). Toll-like receptors (TLRs) serve as pattern-recognition receptors within the innate immune system and recognize exogenous ligands in response to infection (35). Studies have demonstrated that bacteria LPS dramatically initiates an inflammatory response through TLR-4 and directly decreases contractility in cardiomycocytes (2, 25).

Probiotics may prevent cardiovascular diseases such as hypertension through the production of a bioactive peptide that may have angiotensin-converting enzyme inhibitory activities (36). Many recent studies have focused on γ-aminobutyric acid (GABA) production using lactic acid bacteria (LAB) as bacterial cell factories due to the presence of potential GABA bioactive component in foods and pharmaceuticals (4, 16, 19). Consumption of GABA-enriched foods have been reported to depress systolic blood pressure elevation in spontaneously hypertensive rats (SHR) and mildly hypertensive humans (10, 12). Reports have also indicated that probiotics can modulate systemic inflammation, cell proliferation and apoptosis, and such properties may be useful for future immuno-modulatory and cancer prevention strategies (31, 13).

Our present study used PSP (Ipomoea batatas [L.] Lam.) as a fermentation substrate that contains high nutritional value and can easily grow in subtropical areas such as Taiwan, Japan and China. PSP is rich in vitamins, minerals, dietary fiber and non-fibrous carbohydrates and is also an excellent source of the antioxidant anthocyanin (29, 38). Anthocyanins are dietary bioactive compounds with two-fold interests: firstly, there are the technological interests due to their impact on the sensorial characteristics of food products, and secondly for their health-related properties through different biological activities, one of them being their implication on protection from risks of cardiovascular diseases (6, 7).

In our previous study, we found that PSP yogurt (PSPY) fermented with Lactobacillus acidophilus, L. delbrueckii subsp. lactis, and L. gasseri had high GABA activities (38). However, the effects of probiotic-fermented yogurt on hypertension-induced cardiac inflammation and fibrosis were not well understood. This study was designed to investigate the potential benefits of GABA-rich PSPY in the treatment of myocardial fibrosis in SHR with cardiac inflammation, and to elucidate anti-fibrosis effects and TLR-4-related inflammatory pathway blockade of probiotic-fermented PSPY on SHR hearts. It was anticipated that PSPY could not only regulate blood pressure but also anti-inflammatory of hearts, and further prevent the development of cardiac fibrosis.

Materials and Methods

Bacteria Strains and Growth Conditions

Three experimental LAB strains were purchased from the Food Industry Research and Development Institute of Biological Resources Conservation and Research Center, Hsinchu, Taiwan: Lactobacillus acidophilus BCRC 14065 (LA), L. delbrueckii subsp. lactis BCRC 12256 (LDL) and L. gasseri BCRC 14619 (LG). The stock culture was maintained at -80°C in 20% glycerol prior to use. The bacteria were propagated twice in Lactobacilli MRS broth (DIFCO, MD, USA) containing 0.05% L-cysteine each overnight at 37°C before the experimental procedure.
**Preparation of PSP Yogurt**

PSP was acquired from the Taiwan Agricultural Research Institute (Taichung, Taiwan) and stored at 4°C after cleaning with tap water. To prepare PSPY, the potatoes were first peeled and cut into 1-cm slices before steaming at 100°C for 20 min. The cooked spuds were then homogenized with 0.05% α-amylase, 10% skimmed milk powder, 0.05% protease and 3% whey protein added before pasteurization (121°C, 15 min). The three different LAB strains (LA, LDL and LG) were inoculated at a concentration of 10⁹ CFU per ml to the PSP milk and incubated at 37°C for 24 h until 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 SHR and twelve male Wistar Kyoto rats (WKY) were purchased from BioLasco Taiwan Co., Ltd, Taipei, Taiwan, ROC. These animals, aged six weeks, were housed individually in a controlled environment. The rats were maintained on a 12-h dark-light cycle with the lights on from 8 am to 8 pm. The rats were fed with chow pellets (MF-18; Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*. An acclimatization period of one week after delivery by the supplier was allowed before the SHR were randomly distributed into four groups: SHR control (2 ml distilled water), antihypertensive Captopril medicine (15.6 mg/kg, BW/day), 10% PSPY SHR control (2.5 ml distilled water), antihypertensive SHR were sacrificed after 8 weeks of experimental period. The entire experimental procedure was approved by the Institutional Animal Care and Use Committee of Hung Kuang University, Taichung, Taiwan, ROC (Approval No. 96027).

**Masson Trichrome Staining**

The hearts of the animals were excised and were soaked in formalin and covered with wax. Slides were prepared by deparaffinization and dehydration. They were passed through a series of graded alcohols (100, 95 and 75%), 15 min each. The slides were then dyed with Masson trichrome. After gently rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol I and II for 15 min each. At the end, they were soaked with Xylene I and Xylene II. Photomicrographs were obtained using Zeiss Axiophot microscopes (Zeiss, Oberkochen, Germany).

**Tissue Extraction**

The left ventricle was cut into eight 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, 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 s each. The homogenate was placed on ice for 10 min and then centrifuged at 12000 ×g for 40 min. The supernatant was collected and stored at -70°C for western blot analysis.

**Protein Contents**

The protein content of the left ventricle extract was determined using the Bradford protein assay14 using the protein-dye kit (Bio-Rad, Richmond, CA, USA). A commercially available bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) was used as a standard. Changes in absorption were monitored at 595 nm.

**Electrophoresis and Western Blotting**

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). Following that, the electrophoresised proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (0.45-µm pore size) (Millipore, Bradford, 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 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 TNF-α (Cell Signaling, Danvers, MA, USA), TLR-4, phospholatonefκB (p-NFκB), IkB, phospholatonefκB (p-IκB), transforming growth factor-beta (TGF-β) and fibroblast growth factor-2 (FGF-2) (Santa Cruz Biotechnology, 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 three 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) for 1 h and diluted 500-fold in the binding buffer. The filters were then washed three times (10 min each) in the blotting buffer. The immunoblotted proteins were visualized using an enhanced chemiluminescence ECL Western Blotting Luminal Reagent (Santa Cruz) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (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 MgCl₂ in 100 mM Tris-HCl, pH 9.5. The immunoblot with antibody against α-tubulin, prepared using the same procedure, was used as an internal control.

**Statistical Analysis**

All statistical analyses were performed using the 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* < 0.05 were considered statistically significant. The significant differences were stressed with symbols as shown in the tables and figures.

**Results**

**Changes in Cardiac Inflammation-Associated Signaling Pathways of TLR-4 and TNF-α Protein Levels in the Left Ventricle**

Using western blot analysis, TLR-4 was shown to be expressed in the left ventricles obtained in vivo (Fig. 1A). The TLR-4 proteins in the SHR control group were significantly higher (*P* < 0.05) compared with the WKY control group (Fig. 1B). Moreover, SHR-Captopril and the 10% PSPY groups had significantly lower levels of TLR-4 proteins (*P* < 0.05) compared with the SHR control group (Fig. 1B), but the levels were insignificantly different from those in the SHR-100% PSPY group. The TNF-α myocardial cytokine was measured by western blotting to gain an understanding of whether, and to what extent, it is
involved in the hypertensive heart (Fig. 1A). Although the TNF-α protein levels were higher in the SHR control group, this difference was not statistically significant compared with the WKY control group (Fig. 1C). However, TNF-α levels were significantly lower \( (P > 0.05) \) in the SHR-10\% and 100\% PSPY groups compared with the SHR control group.

**Changes in Cardiac Inflammation-Associated Signaling Pathways of \( \text{I} \kappa \text{B} \), p-\( \text{I} \kappa \text{B} \) and p-NF\( \kappa \text{B} \) Protein Levels in the Left Ventricle**

We further determined the effects of \( \text{I} \kappa \text{B} \) and p-\( \text{I} \kappa \text{B} \) in the SHR hearts on PSPY treatment. As shown in Fig. 2B, \( \text{I} \kappa \text{B} \) protein levels in the SHR control group were significantly lower \( (P < 0.05) \) compared with the WKY normal control group. Moreover, the \( \text{I} \kappa \text{B} \) protein levels in the SHR-100\% PSPY group were significantly higher \( (P < 0.05) \) compared with the SHR control group (Fig. 2B), but were insignificantly different from those in the SHR-Captopril and 10\% PSPY groups. The p-\( \text{I} \kappa \text{B} \) protein levels in the SHR control group were significantly higher \( (P < 0.05) \) compared with the WKY normal control group (Fig. 2C). The SHR-Captopril, 10\% and 100\% PSPY groups had significantly lower p-\( \text{I} \kappa \text{B} \) protein levels compared with the SHR control group (Fig. 2C). In addition, the p-NF\( \kappa \text{B} \) protein levels in the SHR control group were significantly higher \( (P < 0.05) \) compared with the WKY normal control group (Fig. 3B). The p-NF\( \kappa \text{B} \) levels were markedly lower \( (P < 0.05) \) in the SHR-100\% PSPY group compared to those in SHR group (Fig. 3B). However, the p-NF\( \kappa \text{B} \) protein products were lower in SHR from Captopril and 100\% PSPY groups compared with the SHR-control group, although these groups showed insignificant differences from the WKY group. An increase in p-NF\( \kappa \text{B} \) protein was triggered in the SHR, but this reaction could be reversed by decreasing \( \text{I} \kappa \text{B} \) phosphorylation and enhancing NF\( \kappa \text{B} \) degradation through the administration of the 10\% and 100\% PSPY in the SHR hearts.

**Changes in Cardiac Fibrosis**

To examine the PSPY effects on cardiac fibrosis in SHR hearts, we performed a histopathological
analysis of ventricular tissues with Masson-trichrome staining. Hearts stained with Masson-trichrome showed minor fibrosis, increased collagen deposition and myofibril disarray in the SHR group in ×200 magnification images, compared with the WKY group (Fig. 4A). Notably, significantly reduced collagen deposition and myofibril disarray were observed in the SHR-PSPY groups (10% and 100% dosages) compared to the SHR group (Fig. 4A).

Effects of PSPY on Cardiac Fibrosis-Associated Protein Expression in SHR Hearts

For further investigation on whether the proteins associated with cardiac fibrosis were influenced by PSPY, TGF-β and FGF-2 were analyzed by western blotting in the left ventricular tissues. As shown in Fig. 4B, we found that the TGF-β protein levels were significantly higher in the SHR group than in the WKY group, but were significantly lower ($P < 0.05$) in the SHR from Captopril, 10% and 100% PSPY groups compared with the SHR-control group (Fig. 4C). Moreover, the FGF-2 protein levels were higher in the SHR group, although that showed insignificant difference to the WKY group (Fig. 4D). In contrast, SHR from the Captopril and 10% PSPY groups had significantly lower protein levels ($P < 0.05$) of FGF-2, compared with the SHR-control group (Fig. 4D).

Discussion

The major findings of this study can be summarized as follows: [1] Minor fibrosis enhancement was observed in SHR hearts, and was associated with significant elevation of the TGF-β and FGF-2 expression levels. In contrast, Captopril and PSPY supplementation reduced cardiac fibrosis significantly concomitant with lowered TGF-β and FGF-2 expression levels. [2] The key components of the inflammation signaling pathway were more activated in the SHR hearts, which were increased in cardiac TLR-4, p-1xB and p-NFκB levels. However, Captopril and PSPY administration significantly decreased the expression of these proteins. [3] Our data supports the hypothesis that cardiovascular inflammation and fibrosis were induced by hypertension in SHR hearts, evidenced by significantly increased fibrotic lesion formation and decreased collagen deposition in cardiac ventricular tissues when compared with WKY rats. Further, Captopril and PSPY could suppress the
Fig. 4. (A) Representative histopathological analysis of cardiac tissue sections stained with masson trichrome in WKY rats, SHR and the SHR groups treated with different doses PSPY (10% and 100% PSPY). The images of myocardial architecture were magnified by 200 times. Collagen accumulation and cardiac fibrosis are shown in blue. (B) The TGF and FGF-2 proteins extracted from the left ventricles of excised hearts in WKY rats, SHR and the SHR groups treated with 10% and 100% PSPY as measured by western blot analysis. (C) and (D) Relative protein quantification of TGF (C) and FGF-2 (D) on the basis of α-tubulin; mean values ± SD are shown (n = 3 in each group). *P < 0.05 compared with the WKY group; #P < 0.05 compared with the SHR-control group.
increases in the levels of TGF-β, FGF-2, TLR-4, p-IkB and p-NFκB (Fig. 5).

The direct relationship between treatment-induced blood pressure reduction and the decrease in some circulating inflammatory markers further confirms hypertension as a potentially pro-inflammatory condition (22). TLR-4 plays an important role in myocardial infarct healing and contributes to LV remodeling and functional impairment following myocardial infarct (35). During inflammation and oxidative stress, TLR-4 is also activated in response to endogenous ligands, such as heat shock protein (HSP) 60 and the alternatively-spliced extra domain A (EDA) of fibronectin, resulting in the release of pro-inflammatory factors such as TNF-α (27, 28). In our studies, we examined the inflammation-related signaling (TLR-4)-(p-NFκB)-p-IκB-TNF-α pathway. The SHR group revealed activation of the TLR-4 protein level, which further resulted in increased phosphorylation and degradation of IκB protein, subsequently leading to p-NF-κB p65 activation. Activated p-NFκB triggered the expression of the downstream target, TNF-α, resulting in the inflammatory responses (1, 37). In contrast, TLR-4, TNF-α and p-IκB proteins presented declined levels in the SHR-10% PSPY group, as well as decreasing p-IκB and p-NFκB p65 proteins in the SHR-100% PSPY group (Figs. 1-3). This suppression of NF-κB nuclear translocation was affected by the inhibitory action of PSPY on TLR-4 and p-IκB expression. Therefore, PSPY has a heart protective effect in the SHR model that may be dependent on reducing the activities of the inflammation-related signaling (TLR-4)-(p-NFκB)-p-IκB-TNF-α pathway.

Cardiac remodeling progresses with an increased level of collagenases immediately after myocardial damage (5). The collagens synthesized by the fibroblasts invade and replace the apoptotic myocytes (17, 26). Accordingly, the accumulated collagens further contribute to the development of ventricular fibrosis and heart failure (17). Kai et al. reported that in WKY rats with suprarenal aortic constrictions, blood pressure elevation induces perivascular inflammation characterized by induction of the monocyte chemotactrant protein-1 (MCP-1) and macrophage infiltration, which triggers TGF-β induction and reactive myocardial fibrosis in the later phase (14, 20). In our findings, hearts positive to Masson-trichrome staining showed minor fibrosis, increased collagen deposition and myofibril disarray in the SHR group (Fig. 4A). However, on eight-week oral administrations of PSPY at both 10% and 100% dosages, less fibrosis was observed in the left ventricular tissues in SHR as well as reduced fibrotic signaling components such as FGF2 and TGB. These findings demonstrated cardiac protective effects of PSPY in SHR by reducing the cardiac fibrosis signaling components.

The western blotting results showed significant effects in the 10% dosage on most of cardiac inflammatory and fibrotic signaling components, which indicated that there were no dose-response effects between 10-fold differences in PSPY concentrations. Administration of GABA in a low dose was considered to effectively decrease blood pressure through a peripheral mechanism (without crossing brain blood barrier) (18). Thus, we speculated that oral administration of 10% PSPY was probably sufficient in providing functional benefits to the cardiovascular disease, based on the assumption that GABA was the major substrate in PSPY which contributed to hypotensive effects and to improve cardiac fibrosis.

Our previous study have demonstrated that a mixture of LA, LGA and LDL strains were the novel GABA-producing LAB (1068.8 ± 21.3 µg/ml) in PSPY (38). In addition, our animal experimentation showed that continuously feeding of PSPY (at both 10% and 100% dosages) for eight weeks significantly reduced systolic and diastolic blood pressure, and average blood pressure of SHR (data not shown). Liu et al. have suggested that the hypotensive effects in SHR may depend on oral administration Lactobacillus-fermented milk, which produces high GABA levels, and could be relative to decreased peripheral vascular resistance (24).

Increased production of reactive oxygen species may exacerbate the chronic inflammation-induced
myocardial fibrosis by elevating the production of pro-inflammatory cytokine mediators (11, 32). Kullisaar et al. proved that the consumption of milk fermented with L. fermentum ME-3 led to antioxidative activity and also the ability to improve anti-atherogenic markers in healthy human subjects (21). The current research has shown that some LAB strains cannot reduce the risk of reactive oxygen species accumulation through food ingestion but can degrade the superoxide anion and hydrogen peroxide (23). Additionally, some in vitro studies have demonstrated benefit effects of anthocyanins such as antioxidative radical-scavenging ability and inhibitory effects on Angiotensin I-converting enzyme (ACE) that could contribute to the antihypertensive effect (33, 34). Our previous study has also indicated that the fermentation procedure could further significantly elevate anthocyanin content and antioxidative activities in the PSPY with LA, LDL and LG strains (38). A recent study demonstrated that anthocyanins in the purple carrot juice contribute to antioxidant and anti-inflammatory properties to improve cardiovascular structure and function (30). High levels of anthocyanins, antioxidant activities and GABA concentrations in probiotic-fermented PSPY might have a protective role in myocardial fibrosis through the suppression of TLR-4-related inflammatory pathway.

In conclusion, PSPY reveals the effects on cardiac protection by reducing fibrosis and fibrotic signaling molecules in the left ventricle tissues of SHR as well as decreased cardiac inflammatory signaling components in SHR. These findings may provide clues to understanding the cardiac protective mechanism of PSPY on attenuate inflammation and fibrosis and suggest health-care potentials of GABA-rich PSPY in treating hypertension patients with cardiovascular disease.

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