Li-Fu formula prevents the IL-6 mediated cardiac hypertrophy in hyper cholesterol-fed hamsters

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ABSTRACT

Hypercholesterolemia diets are considered as major sources to cause cardiac hypertrophy. This study intends to evaluate the effects of Li-Fu formula on cardiac hypertrophy induced by hypercholesterolemia diet. Twenty-four male Golden Syrian hamsters at 3 months of age were randomly divided into Control, Cholesterol and Li-Fu formula groups and fed with different experimental diets for 2 months. Histopathological analysis and western blotting were performed to measure the myocardial architecture, and expressions of different cardiac hypertrophy-associated molecules in the excised left ventricle from hamsters. The ratios of Whole heart weight (WHW)/Body weight (BW) and Left ventricle weight (LVW)/BW were significantly higher in the Cholesterol group but significantly lower in the Li-Fu formula group. The protein levels of both ANP and BNP were significantly increased in the Cholesterol group but significantly reduced in the Li-Fu formula group. Additionally, significantly increased interleukin (IL)-6, STAT3, MEK5, p-ERK5 and non-cardiomyocyte proliferate signal molecules such as p-MEK and p-ERK, were detected in the Cholesterol group but significantly reduced in the Li-Fu formula group. Notably, no significant variations of inflammatory signaling molecules, including p-P38 and p-JNK, were detected in all groups. Our experimental results demonstrate the significant reductions of cardiac hypertrophy and related eccentric hypertrophy

signaling, non-cardiomyocyte proliferate signaling in the excised left ventricle of hamsters from the Li-Fu formula. We suggested the protective effects of Li-Fu formula on cardiac hypertrophy that may be useful in prevention or treatment of hypertrophy-associated cardiovascular diseases.

Key Words: hypercholesterol, hypertrophy, Li-Fu formula, heart

Introduction

Hypercholesterol diets are the major sources to cause cardiac hypertrophy (1). Cardiac hypertrophy is recognized as a cardiac adaptive response to any stress that can exist in a state of compensation or progress to a decompensated state over time (2). Prolonged hypertrophy of the cardiomyocytes is demonstrated as the main cause of sudden cardiac death (3). A number of studies indicated that various diseases have been associated with cardiac hypertrophy including occlusive atherosclerotic coronary heart disease (CHD), associated myocardial infarction (MI), heart failure hypertension, endocrine disorders, toxicants, and bacterial endocarditis (4-7).

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are known as the cardiac hormones in normal adults that were secreted by the atria and ventricles. Higher levels of ANP and BNP expression are expressed in the fetal ventricles than adult ventricles (8). Cardiac ANP and BNP levels are increased in myocardial infarction of animal models (9), heart failure (10), hypertrophy (11), and also in human cardiac diseases (12). Increased expressions of ANP and BNP are observed in ventricular during the molecular process of cardiac hypertrophy, which are recognized as markers of the induction of the embryonic gene program in ventricular hypertrophy (13).

Interleukin (IL)-6 is known as a potent hypertrophic factor of cardiomyocytes (14-15). The IL-6 receptor system consists of various signaling pathways including inflammatory related p38 MAPK, and hypertrophy involved STAT1-STAT3 heterodimer pathway, STAT3 homodimer pathway, and non-cardiomyocyte proliferative related MAPK extracellular signal regulated kinase (ERK)s pathway that are activated by the dimerization of gp130 (16-19). The activation of STAT3-dependent signaling pathway by gp130 was reported to promote cardiac myocyte hypertrophy (20), herein the STAT1 and the STAT 3 were shown to be chronically phosphorylated in the failing heart (21). Moreover, the ERK5 molecule plays a critical role in post-natal eccentric hypertrophy of the heart (9, 22). ERK5 and its upstream MAPK-kinase 5 (MEK5) reveals a specific role in transduction of cytokine signals that regulate serial sarcomere assembly and in the induction of eccentric cardiac hypertrophy resulting in dilated cardiomyopathy and sudden death (22). Therefore, it is crucial to investigate the pathologic role of IL-6-MEK5-ERK5 signaling pathway under cardiac hypertrophy. Additionally, various molecules have been elucidated responsible for the development of cardiac hypertrophy, including mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and calcineurin pathway (23). The extracellular-regulated kinase (ERK), the c-Jun

N-terminal kinases (JNK), and the p38 MAPK cascades (p38) enrolled in the MAPK pathway also play crucial roles in the development of cardiac hypertrophy (24).

To avoid the side effects by administration of western drugs, growing studies were performed to investigate the natural products for the cardiac protection that have been used as drugs or diet supplements for a long history in many medical-experiences. Recent studies reported the cardioprotective effect of various oriental herb extracts or dietary supplements including Fructus Crataegi, Salvia miltiorrhiza and Astragali radix. The quercetin is the main ingredient in Fructus crataegi that has been demonstrated as an anti-inflammatory substance by inhibiting TNF-α release from macrophages and recognized to have cardiac protective effect (25,26). Salvia miltiorrhiza is mainly composed of sodium tanshinone IIA sulfonate (STS), a derivative of tanshinone IIA that can reduce myocardial infarct size and prolong the survival cardiac cell in rabbit and human (15,27,28). Astragali radix contains many isoflavones, isoflavonoids, and many saponins, which have been demonstrated to have protective effects on heart by reducing inflammation, oxidant and cardiac ischemia-reperfusion injury (29-34) In our recent publication, we also demonstrated the protective effect of Li-Fu formula composing of Celery, Black fungus, Mushroom, Saliva miltior rhiza, Crataegi cuneata, and Astragali radix on cardiac apoptosis (35).

To further understand the effects and possible mechanisms of Li-Fu formula on cardiac hypertrophy, we performed the histopathological analysis and Western blotting assay to examine the expression of hyper-trophic associated molecules in the cardiac tissues from hamsters that were fed with hypercholesterol diets. We suggest the cardiac protective effects of Li-Fu formula by reducing the cardiac hypertrophy.

Material and Methods

Animals and diet

A total of 24 male Golden Syrian hamsters weighting 135 to 170 gram at the age of 8 weeks were purchased from National Laboratory Animal Center, Taipei, Taiwan, and housed in an animal room at 22 ± 2 °C with a 12/12 h light−dark cycle under supervision of Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan. Hamsters were acclimatized for 2 weeks while receiving free access to water and were fed chow diet (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) ad libitum. The hamsters were then randomized into 3 groups as control, cholesterol and Li-Fu formula groups and switched to experimental diets. The control, cholesterol and Li-Fu formula groups received chow diet, chow diet with 0.2% cholesterol (Sigma, Saint Louis Mo, USA), and chow diet with 0.2 % cholesterol and 2% Li-Fu formula for 8 weeks, respectively. Celery and Black fungus are obtained from common supermarket and Mushroom, Saliva miltior rhiza, Crataegi cuneata and Stragali radix are purchased from traditional Chinese pharmacy. The Li-Fu formula was firstly created and provided by Dr Li-Fu Chen, China Medical University, Taichung, Taiwan. To make Li-Fu formula, every component of desired weight was crushed and mixed with a blender, then placed in 1000ml distilled water and boiled for 1 h under reflux. The resultant solution was

divided into several parts and stored in a –80 °C freezer for further use (35). The Li-Fu formula is composed of Celery, Black fungus, Mushroom, Saliva miltior rhiza, Crataegi cuneata, and Astragali radix as shown in Table 1 and the experimental dietary composition is shown in Table 2. The Ambient temperature was maintained at 25°C. Diets were prepared weekly and stored at -80°C. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan. Food intake and food spillage were measured daily, and body weight was recorded every 3 days.

Cardiac characteristics

Three groups of hamsters at age of 8-9 month old were weighed and decapitated after receiving 8 weeks of experimental diets. The hearts of animals were excised and cleaned with distilled H_2O. The left and right atrium and ventricle were separated and weighed. The body weight (BW), left ventricle weight (LVW), the ratios of the whole heart weight (WHW) to body weight (BW) and the ratios of the left ventricular weight (LVW) to body weight (BW), were measured and calculated.

Hematoxylin-eosin staining

The hearts of 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 minutes of each. The slides were then dyed with hematoxylin. After gently rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol I and II for 15 minutes each. At the end, they were soaked with Xylene I and Xylene II. Photomicrographs were obtained using Zeiss Axiophot microscopes.

Tissue Extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a PBS buffer (0.14 M NaCl, 3 mM KCl, 1.4 mM KH_2PO_4, 14 mM K_2HPO_4) at a ratio of 100 mg tissue/0.5ml PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 g for 30 min. The supernatant was collected and stored at -70°C for further experiments. Protein concentration was determined using a BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA) and were quantified by absorbance at 595 nm using a spectrophotometer (Beckman Coulter, Palo Alto, CA, USA).

Electrophoresis and Western Blot

The tissue extract samples were prepared as described above. Sodiumdodecyl

sulfate-polyacrylamide gel electrophoresis was performed with 10% polyacrylamide gels. The samples were electrophoresed at 140 V for 3.5 hours and equilibrated for 15 min in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (V/V) methanol. Electrophoresed proteins were transferred to nitrocellulose membranes (Amersham, Hybond-C Extra Supported, 0.45μm pore size) with a Bio-Rad Scientific Instruments Transphor Unit at 100 mA for 14 h. Nitrocellulose membranes were incubated at room temperature for 2 hours in blocking buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) fetal bovine serum. Antibodies including ANP, BNP, IL-6, STAT3, MEK5, p-EKR5, MEK, p-MEK, p-ERK, p-P38, JNK, p-JNK and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted to 1:200 in antibody binding buffer containing 100 mM Tris-HCL, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 and 1% (v/v) fetal bovine serum. Incubations were performed at room temperature for 3.5 hours. The immunoblots were washed three times in 50 ml blotting buffer for 10 min and then immersed in the second antibody solution containing horseradish peroxidase (HRP) conjugated goat anti-hamster IgG (Promega Corp., Madison, WI, USA) for 1 hour that was diluted 1000-fold in binding buffer. The immunoblots were then washed in blotting buffer for 10 min three times. Pierce's Supersignal West Dura HRP Detection Kit (Pierce Biotechnology Inc., Rockford, IL) was used to detect antigen-antibody complexes.

The blots were scanned and quantified by densitometry (Appraise, Beckman-Coulter, Brea, California, USA).

Statistical Analysis

All of the statistical analyses were performed using SPSS 10.0 software (SPSS Inc., Chicago, IL). Three independent experiments were repeated. Statistical analyses were performed using the analysis of variance plus posterior multiple comparison test to test the difference. The data between two experimental animal groups was compared by Student's t-test for two independent samples. In all cases, a difference at $P<0.05$ was considered statistically significant.

RESULTS

Experimental diets and cardiac characteristics

To investigate the effect of Li-Fu formula on hypertrophy in cardiac cells, we examined the body weight and cardiac characteristics. Firstly, Li-Fu formula was prepared as described in materials and methods and the compositions of the Li-Fu formula was shown in Table 1. Table 2 presents the ingredients of experimental diets for different groups of hamsters. Body weight (BW), left ventricle weight (LVW), the ratios of whole heart weight (WHW) to body weight (BW) and the ratios of left ventricular weight (LVW) to body weight (BW) of hamsters from Control, Cholesterol and Li-Fu formula groups were detected (Table 3). The ratios of WHW/BW and LVW/BW were significantly higher in hamsters of the Cholesterol group compared to the Control group. Notably, the ratios of WHW/BW and LVW/BW were significantly reduced in the hamsters from the Li-Fu formula group compared to the Cholesterol group (Table 3).

Cardiac architecture changes

To further confirm the effect of Li-Fu formula on the cardiac hypertrophy, we did a cross section of whole heart and histopathological analysis of ventricular tissue stained with hematoxylin and eosin. We found that ventricular wall thickness

significantly increased in the Cholesterol group but significantly decreased in the Li-Fu formula group (Fig. 1A). The ventricular myocardium in the Control group showed normal architecture with normal interstitial space. In contrast, the abnormal myocardial architecture and the increased interstitial space were observed in the Cholesterol group that shows structural disorganization and cardiomyocyte disarray but significantly decreased in the Li-Fu formula group in 400X magnification images (Fig. 1B). Moreover, the protein levels of both ANP and BNP were significantly increased in hearts of the Cholesterol group compared to the Control group. In contrast, significantly reduced ANP and BNP protein expressions were detected in hearts of hamsters from the Li-Fu formula group (Fig. 2).

Effect of Li-Fu formula on cardiac hypertrophy associated signaling pathways

In order to identify the hyper-trophic factor IL-6, signal transducer and activator of transcription STAT-3 and mitogen-activated protein kinase/ERK (MEK) signaling pathways associated with the cardiac hypertrophy induced by hypercholesterol diet, the protein products of IL-6, STAT3, MEK5, and p-ERK5 were measured by western blotting. In hearts of the Cholesterol group, the protein products of IL-6, STAT3, MEK5, and p-ERK5 showed significant increase potency compared to the hearts of the Control group (Fig. 3). However, significantly decreased IL-6, STAT3, MEK5,

and p-ERK5 protein expression was observed in hearts of the Li-Fu formula group (Fig 3). We further detected the protein levels of MEK and p-MEK. As shown in figure four, significantly increased MEK and p-MEK protein levels were detected in hearts of the Cholesterol group compared to the Control group (Fig. 4). Notably, significantly decreased p-MEK was observed in hearts of the Li-Fu formula group compared to the Cholesterol group (Fig 4). Additionally, significantly increased p-ERK protein was detected in hearts of the Cholesterol group compared to the Control group. In contrast, significantly reduced p-ERK protein was observed in hearts of hamsters from the Li-Fu formula group compared to those from the Cholesterol group (Fig. 5). However, no significant variations in p-P38 and p-JNK protein levels were detected between hearts of the Control and the Cholesterol groups or the Cholesterol and the Li-Fu formula groups (data not shown).

DISCUSSION

Hypercholesterolmia diets have been recognized as the major sources to cause cardiac hypertrophy and associated with numbers of heart diseases (4,5,7,32). Because of the side effects of western drugs in treatment of cardiac diseases, the investigations of natural product such as dietary supplements or oriental herbs on cardiac protection are performed. In the current study, we intend to elucidate the effect of a formula composed of dietary supplements and oriental herbs on cardiac hypertrophy. Our experimental results indicated the significant reduction of the WHW/BW and LVW/BW ratios in hamsters from Li-Fu formula group compared to those from the Cholesterol group. Moreover, the hypertrophic marker protein such as ANP, BNP, eccentric hypertrophic related factors such as IL-6, STAT3, MEK5, p-ERK5, p-MEK and p-ERK were significantly increased in the Cholesterol group whereas significant reduction of all these proteins were observed in the Li-Fu formula group.

The interleukin (IL)-6 is known as a pleiotypic factor that has been associated with various cardiac diseases (14,36,37). Elevated IL-6 mRNA is observed in patients of cardiac hypertrophy with hypertrophic cardiomyopathy (37). As figure 7 shows, various signaling molecules, including p38 MAPK, STAT1-STAT3 heterodimer pathway, STAT3 homodimer pathway, and MAPK extracellular signal regulated

kinase (ERK)s pathway, were induced by IL-6 receptor signaling systems and contributed to the cardiac hypertrophy.[1,9,17,18-20,22] In our experimental results, significant elevation of IL-6 expression was detected in the excised ventricle of hamsters from the Cholesterol group as well as those hypertrophic related signaling molecules including STAT3, MEK5, p-ERK5. Notably, the significant reduction of these hypertrophic factors and signaling molecules were detected in the excised ventricle of hamsters from the Li-Fu formula group. To further clarify the involved signaling pathway, we further examined the MAPK pathway that is important in cardiac hypertrophy and consists three major cascades including the non-cardiomyocyte proliferative extracellular-regulated kinase (ERK), and the inflammatory related c-Jun N-terminal kinases (JNK), and the p38 MAPK cascades (p38) (24). As revealed in current study, significant increase of phosphorylated ERK (p-ERK) was observed in the excised ventricle of hamster from the Cholesterol group and the p-ERK level was significantly reduced in the excised ventricle of hamster from the Li-Fu formula group. Moreover, higher increase of p-MEK, the upstream kinase activator of EKR, was also detected in hamsters from the Li-Fu group compared to the Cholesterol group. However, no significant differences in protein levels of p-P38 and p-JNK were detected between hamsters from the Cholesterol and the Li-Fu formula groups. These findings suggest that Li-Fu formula has the effect

against cardiac hypertrophy via attenuation of non-cardiomyocyte proliferation related p-ERK cascade but not P38 or JNK cascade.

Because of the moderated side effects than western drugs, more than half of the population in the world relies on traditional medicine for therapeutic needs. Indeed, herbal remedies and alternative medicines are used throughout the world and in the past herbs often represented the original sources of most drugs (38,39,40). The Li-Fu formula was firstly created by Dr. Li-Fu Chen, China Medical University, Taichung, Taiwan, and composed of various dietary supplements and oriental herbs, including Celery, Black fungus, Mushroom, Saliva miltior rhiza, Crataegi cuneata, and Astragali radix that were routinely used as traditional medicine in oriental worlds. For instance, a major ingredient of Li-Fu formula, Salvia miltiorrhiza, is known as "Danshen" and mainly composed sodium tanshinone IIA sulfonate (STS), a derivative of tanshinone IIA that is also known to protect cardio-vascular ischemia-reperfusion and oxidant injuries (15,27,28,30,32,33,34,39,40). To elucidate the effect and possible mechanism of Li-Fu formula on hypercholesterolmia induced cardiac hypertrophy, we performed the histopathological analysis and western blotting to measure the myocardial architecture, and expression of different cardiac hypertrophy associated molecules in the excised left ventricle from hamsters. Notably, markedly reduced ratios of

WHW/BW and LVW/BW were observed in hamsters from the Li-Fu formula group compared to those from the Cholesterol group. These findings did suggest the protective effects of Li-Fu formula on cardiac hypertrophy.

In the world, more than half of the population relies on traditional medicine for therapeutic needs either by stewing or solution extracting (39-41). Although the precise mechanism of most herbal medicine or dietary supplement has not been fully understood, the experience of the traditional use over the years cannot be neglected". Altogether, our experimental results revealed that Li-Fu formula, the traditional oriental herbs and diet supplements formula, have significant protective effects against cardiac hypertrophy. Besides the attenuated expression of ANP and BNP, the effect against cardiac hypertrophy of Li-Fu formula is probably via the reduction of eccentric hypertrophy related IL-6 receptor pathway and non-cardiomyocyte proliferation involved ERK signaling cascade but not JNK and P38 cascades. Therefore, the Li-Fu formula could provide an alternative regimen for the prevention or treatment of cardiac hypertrophy.

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Figure legends

Figure 1. Cardiac cross sections and cardiomyopathic changes in hamsters of control, cholesterol and Li-Fu formula groups. (A) The cross section of whole heart in the three groups. Arrows indicate that the left ventricular lumen diameters increased in the cholesterol group but decreased in the Li-Fu formula group. (B) Representative histopathological analysis of cardiac tissue sections with Hematoxylin and eosin staining in hamsters of control, cholesterol and Li-Fu formula groups. The images of myocardial architecture were magnified by 100 times.

Figure 2. (A) The representative protein products of ANP and BNP extracted from the left ventricles of excised hearts in hamsters of Control, Cholesterol and Li-Fu formula groups were measured by Western Blotting analysis. (B)(C) Bars represent the relative protein quantification of ANP and BNP on the basis of α-tubulin. All bars indicate mean values±SD (n=6 in each group). $**P<0.01$, significant differences between Control and Cholesterol group. $\#P<0.05$ *and* $\#\#P<0.01$, significant differences between Cholesterol and Li-Fu formula groups.

Figure 3. (A) The representative protein products of IL-6, STAT3, MEK5 and p-ERK5 extracted from the left ventricles of excised hearts in hamsters of Control, Cholesterol

and Li-Fu formula groups were measured by Western Blotting analysis. (B)(C)(D)(E) Bars represent the relative protein quantification of IL-6, STAT3, MEK5 and p-ERK5 on the basis of α-tubulin. All bars indicate mean values±SD (n=6 in each group). **P<0.01, significant differences between Control and Cholesterol group. ##$P<0.01$, significant differences between Cholesterol and Li-Fu formula groups.

Figure 4. (A) The representative protein products of p-MEK and MEK extracted from the left ventricles of excised hearts in hamsters of Control, Cholesterol and Li-Fu formula groups were measured by Western Blotting analysis. (B)(C) Bars represent the relative protein quantification of p-MEK and MEK on the basis of α-tubulin. All bars indicate mean values±SD (n=6 in each group). **P<0.01, significant differences between Control and Cholesterol group. ##$P<0.01$, significant differences between Cholesterol and Li-Fu formula groups.

Figure 5. (A) The representative protein product of p-EKR extracted from the left ventricles of excised hearts in hamsters of Control, Cholesterol and Li-Fu formula groups were measured by Western Blotting analysis. (B) Bars represent the relative protein quantification of p-ERK on the basis of α-tubulin. All bars indicate mean values±SD (n=6 in each group). **P<0.01, significant differences between Control

and Cholesterol group. *##P<0.01*, significant differences between Cholesterol and Li-Fu formula groups.

Figure 6. Our proposed hypothesis that cardiac IL-6, MEK-5-ERK-5 and STAT3 hypertrophic pathways and MEK1/2-ERK1/2 non-cardiacmyocyte proliferative pathway are more activated in hyper cholesterol-fed hamaster hearts. The eccentric hypertrophy related pathway, IL-6 related MEK5-ERK5 pathways and MEK1/2-ERK1/2 non-cardiacmyocyte proliferative pathway may play a part of role for developing eccentric cardiac hypertrophy and pathological changes in hyper cholesterol-fed hamaster hearts. Dash lines represent possible theoretical pathways but is still unconfirmed. Up arrows and down arrows on the right side represent increases and decreases, respectively.