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Effects of Garlic Oil on Interleukin-6 Mediated Cardiac Hypertrophy in Hypercholesterol-Fed Hamsters

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Abstract

Hypercholesterol diets are the major causes of cardiac hypertrophy and various cardiac disorders. The purpose of this study is to evaluate the effects of garlic oil on cardiac hypertrophy induced by hypercholesterol diets. Golden Syrian hamsters were fed with 2% cholesterol or 2% cholesterol plus 1% garlic oil for 2 months. Heart architecture changes were measured by hematoxylin-eosin staining and the molecular mechanism was determined by western blotting. Garlic oil reduced whole-heart weight to bone weight ratio, and left ventricle weight to bone weight ratio in the cholesterol-fed group. Moreover, the garlic oil group showed significantly reduced interleukin-6, phosphorylated (p)-extracellular signal-regulated kinase-5, p-mitogen-activated protein kinase-5, calcineurin, nuclear transcription factor of nuclear factor of activated T-cells-3 and p-GATA binding protein 4 when compared with the cholesterol group. However, no changes were observed in gp-130, signal transducer and activator of transcription-3, p-P38 and p-Jun N-terminal kinases protein levels in all groups. The results show that garlic oil may be useful in the treatment of hypertrophy-associated cardiovascular diseases.

Key Words: garlic oil, heart, hypercholesterol, hypertrophy

Introduction

Cardiac (ventricular) hypertrophy is associated with increased cardiac myocyte cell volume, enhanced protein synthesis, changes in gene transcription and translation, and increased myofibrillar assembly (11,

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36). Cardiac hypertrophy often occurs in response to increased hemodynamic loads arising from a variety of conditions including hypertension, myocardial infarction, endocrine disorders and hypercholesterol diets (5, 6, 11, 14, 20). Prolonged hypertrophy frequently results in myocyte disarray and apoptosis as well as ventricular fibrosis, resulting in progression in heart failure and sudden death (14).

A plethora of signaling cascades have been implicated in the activation of the hypertrophic gene program and cardiomyocyte growth. Interleukin (IL)-6 is known as a potent hypertrophic factor of cardiomyocytes (20). The IL-6 receptor system is associated with various signaling pathways such as the p38-mitogen-activated protein kinase (MAPK) pathway, signal transducer and activator of transcription 3(STAT1)-signal transducer and activator of transcription 3(STAT3) heterodimer pathway, STAT3 homodimer pathway, and the MAPK extracellular signal-regulated kinase (ERK)s pathway that are activated by the dimerization of gp130 (18, 19, 21, 30). The activation of STAT3-dependent signaling pathway by gp130 was reported to promote cardiac myocyte hypertrophy (22). Herein, the STAT1 and the STAT3 were shown to be chronically phosphorylated in the failing heart (28).

Moreover, the ERK5 molecule plays a critical role in post-natal eccentric hypertrophy of the heart (2, 29). 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 (29). Therefore, it is crucial to investigate the pathologic role of the IL-6-MEK5-ERK5 signaling pathway under cardiac hypertrophy. Additionally, various molecules have been elucidated that are responsible for the development of cardiac hypertrophy, including MAPK, phosphoinositide 3-kinase (PI3K) and calcineurin pathway (20).

The ERK, the c-Jun N-terminal kinases (JNK), and the p38 MAPK cascades (p38) enrolled in the MAPK pathway play crucial roles in the development of cardiac hypertrophy (33). Another pathway that has received attention is mediated by the Ca^{2+}-calmodulin-activated phosphatase, calcineurin (Protein Phosphatase 2B, PP2B) (40). Once activated, calcineurin directly binds to and dephosphorylates nuclear factor of activated T-cells-3 (NFAT-3) transcription factor in the cytoplasm, permitting its translocation to the nucleus where dephosphorylated NFAT-3 further interacts with GATA binding protein 4(GATA-4) transcription factor to form the complex that participates in the development of myocardial hypertrophy and the expressions of hypertrophy response genes (25, 26).

At the ends of intracellular signaling pathways are re-expression of the so-called fetal gene program. This program includes increased expression of β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Plasma levels of ANP and BNP are markedly elevated in heart failure (27) after myocardial infarction (MI) (13) and are powerful predictors of ventricular dysfunction and mortality (23). Moreover, within the heart tissue, expression of both ANP and BNP is reportedly up-regulated in animal models of MI and heart failure (3, 4, 16, 24, 34, 35, 37).

To avoid the side effects in the administration of chemical drugs, growing studies were performed in the past years to investigate natural products for cardiac protection that have been used as drugs or diet supplements in many medical experiences. Consumption of garlic (*Allium sativum* L) has been reported to have a variety of cardio-vascular effects, including reduction in plasma cholesterol, leading to reports that garlic oil can prevent fat-induced hyperlipidemia. It has also been reported that ingestion of garlic has resulted in hypolipidemia and inhibition of atherogenesis (1, 9, 10, 17).

In the current study, to understand the effects and possible mechanisms of garlic oil on cardiac hypertrophy, we performed histopathological analysis to examine the expression of hypertrophy-associated molecules in the cardiac tissues in hamsters that were fed with a hypercholesterol diet. Our data suggested cardiac protective effects of garlic oil by reducing cardiac hypertrophy.

Materials and Methods

Animals and Diet

A total of 24 male Golden Syrian hamsters weighing 135 to 170 gram at the age of 8 weeks were purchased from the National Laboratory Animal Center (Taipei, Taiwan, ROC) and were used in this study. 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 garlic oil groups and were switched to the experimental diets. The control, cholesterol and garlic oil groups received chow diet, chow diet with 2% cholesterol (Sigma, Saint Louis MO, USA), and chow diet with 2% cholesterol and 1% garlic oil for 8 weeks, respectively. 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, ROC). Food intake and food spillage were measured daily,

and body weight (BW) was recorded every 3 days.

Cardiac Characteristics

Three groups of hamsters at the age of 8-9 months old were weighed and decapitated after receiving 8 weeks of experimental diets. The hearts of the animals were excised and cleaned with distilled H_2O. The left and right atrium and ventricle were separated and weighed. The right tibias were also separated and tibia length was measured by the electronic digital vernier caliper for correcting the whole heart weight (WHW). The BW, left ventricle weight (LVW), the ratios of the WHW to BW and the ratios of the left ventricular weight (LVW) to BW, were measured and calculated.

Hematoxylin-Eosin Staining

The hearts of animals were excised and were soaked in formalin and covered with wax. Slides (1 mm) were prepared with 0.2 μm-thick tissue sections by deparaffinization and dehydration. They were passed through a series of graded alcohols (100%, 95% and 75%), 15 min of each step. The slides were then stained with hematoxylin and eosin. After gently rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol I and II for 15 min each. They were finally 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/0.5 ml 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.

Western Blot

Tissue extract samples were prepared as described above. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 10% polyacrylamide gels. The samples were electrophoresed at 140 V for 3.5 hand 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 Hybond-C Extra Supported nitrocellulose membranes (0.45-μm pore size; Amersham, PA, USA) with a Bio-Rad Scientific Instruments Transphor Unit at 100 mA for 14 h. Nitrocellulose membranes were incubated at room temperature for 2 h in blocking buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) fetal bovine serum. Antibodies directed against ANP, BNP, IL-6, STAT3, p-EKR5, p-MEK, p-ERK, p-P38, p38, p-JNK and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted to 1:200 in an 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 h. 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 h that was diluted 1,000-fold in the binding buffer. The immunoblots were then washed three times in blotting buffer for 10 min each. Pierce's Supersignal West Dura HRP Detection Kit (Pierce Biotechnology Inc., Rockford, IL, USA) was used to detect antigen-antibody complexes. The blots were scanned and quantified by densitometry (Appraise, Beckman-Coulter, Brea, CA, USA).

Statistical Analysis

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

To investigate the effects of garlic oil on cardiac hypertrophy and architecture changes in cardiac cells, we examined the BW and cardiac characteristics, including the LVW, the ratios of the WHW to BW, and the ratio of the LVW to BW of hamsters were compared with the control. In the cholesterol and garlic oil-treated groups, the WHW and the ventricular heart weight were significantly higher in the cholesterol group as compared to the control group. Notably, the ratios of WHW/BW and LVW/BW were reduced in the hamsters from the garlic oil group as compared to the cholesterol group (Table 1).

To further identify the cardiac architecture changes during cardiac hypertrophy, whole hearts were cross sectioned and then stained with H & E to analyze the ventricular tissues. Ventricular wall thickness was found to be significantly increased in the cholesterol group but in the garlic oil group it was decreased (Fig. 1A). It was observed that the cholesterol-treated groups displayed abnormal myocardial architecture and increased interstitial space (Fig. 1B). However this abnormal myocardial architecture and the interstitial space increase were not observed in the garlic

Table 1. Whole heart weight, left heart weight, whole heart weight/body weight, left heart weight/body weight ratio

	Control	2% Cholesterol	2% Cholesterol + 1% Garlic oil
Whole heart weight (mg)	410.63 ± 36.10	423.1 ± 21.92	394.53 ± 27.14
Left heart weight (mg)	290.5 ± 8.82	292.0 ± 12.98	269.30 ± 4.16
Whole heart weight/Body weight	3.83 ± 0.02	4.10 ± 0.01*	3.89 ± 0.02#
Left heart weight/Body weight	2.71 ± 0.01	2.83 ± 0.04*	2.70 ± 0.05#

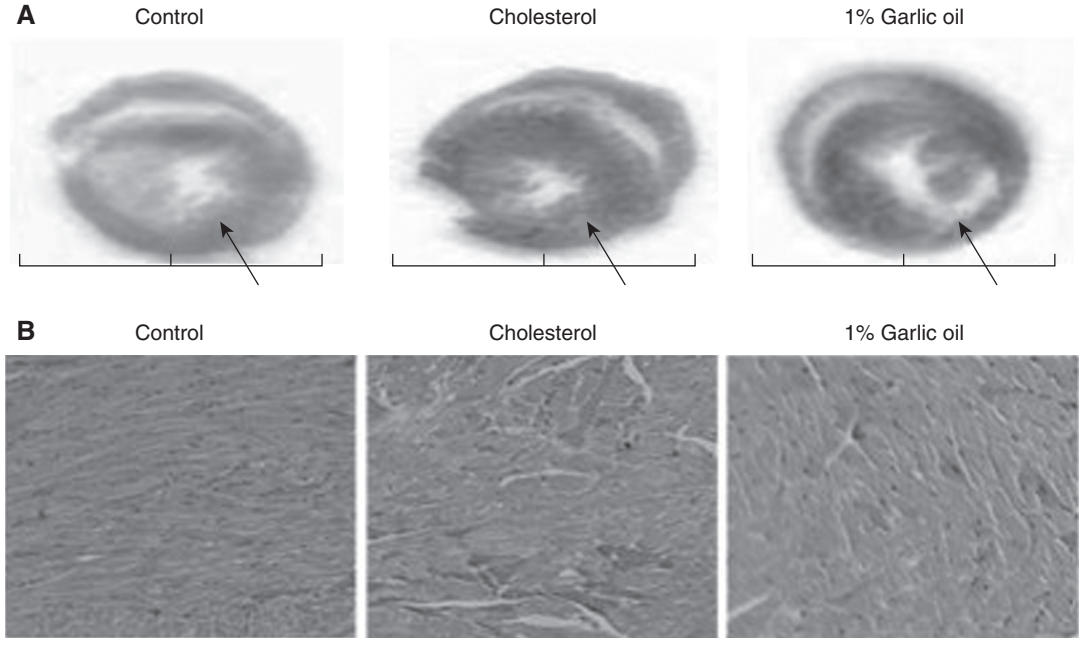

Fig. 1. Cardiac cross sections and cardiomyopathic changes in hamsters of the control, cholesterol and garlic oil groups. (A) Cross sections of whole hearts of the hamsters. Arrows indicate that thickness of the left ventricular wall increased in the cholesterol group but decreased in the garlic oil group. (B) Representative histopathological analysis of cardiac tissue sections with hematoxylin and eosin staining. The images of the myocardial architecture were 100× magnification.

oil group.

Next we analyzed how the hypercholesterol diet affected the cardiac hypertrophy-associated hormones ANP and BNP. ANP and BNP were significantly increased in the cholesterol group compared with the non-cholesterol treated control group, whereas ANP and BNP expression were significantly reduced in the hearts of the garlic oil group (Fig. 2A).

We further examined activation of hypertrophy-related transcription factors GATA-4 and NFAT-3 (Fig. 3). Calcineurin, NFAT3 and phosphorylated (p)-GATA4 were significantly increased in the cholesterol group compared with the control group whereas garlic oil treatment significantly reduced calcineurin, NFAT3 and p-GATA4 protein expression (Fig. 3). The hypertrophy-associated IL-6 signaling pathway was also analyzed by western blotting. The heart tissues of the cholesterol group showed increased IL-6 and p-ERK5 expression as compared to those of the control group (Fig. 4). However, significantly decrease of IL-6 and p-ERK5 protein expression was observed in hearts of the garlic oil group (Fig. 4). STAT3 and gp130 protein levels remained unchanged in the cholesterol group (Fig. 4).

The cardiac hypertrophy associated MEK signaling pathways induced by hypercholesterol diets and the levels of the components of c-JUN N-terminal kinase (c-JNK), ERK, and p38 were next examined. Significant increased of MEK and p-MEK protein levels were detected in the hearts of the cholesterol group as compared to those of control group (Fig. 5). Notably, a significant decrease of p-MEK was observed in hearts of the garlic oil group as compared to those of the cholesterol group (Fig. 5). Additionally, significant increases of the p-ERK protein were detected in the cholesterol group as compared to the control group whereas a significant reduction of p-

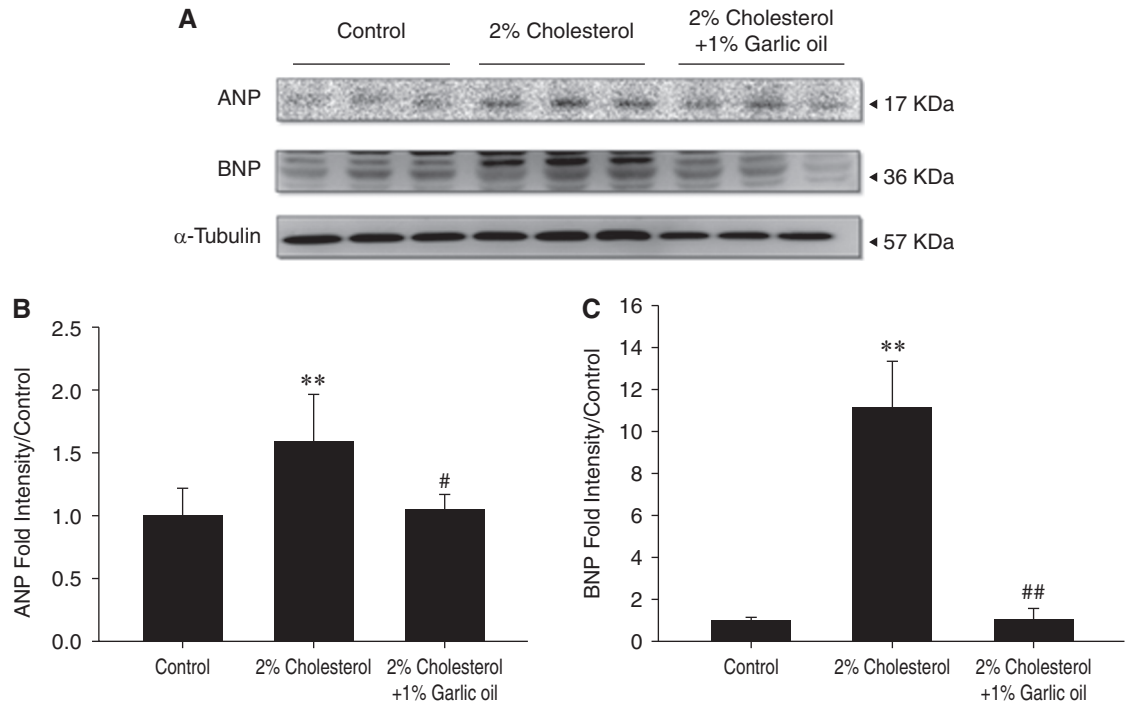

Fig. 2. Effects of garlic oil on the expression of hypertrophic protein markers. (A) Levels of ANP and BNP extracted from the left ventricles of hamsters in the control, cholesterol and garlic oil groups were measured by western blot analysis. (B & C) Quantification of the western blots in (A). Relative protein levels of ANP and BNP were normalized to that of α-tubulin. All bars indicate mean ± SD; n = 6 in each group. $^{**}P < 0.01$ indicates significant differences between the control and cholesterol groups. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ denote significant differences between the cholesterol and garlic oil groups.

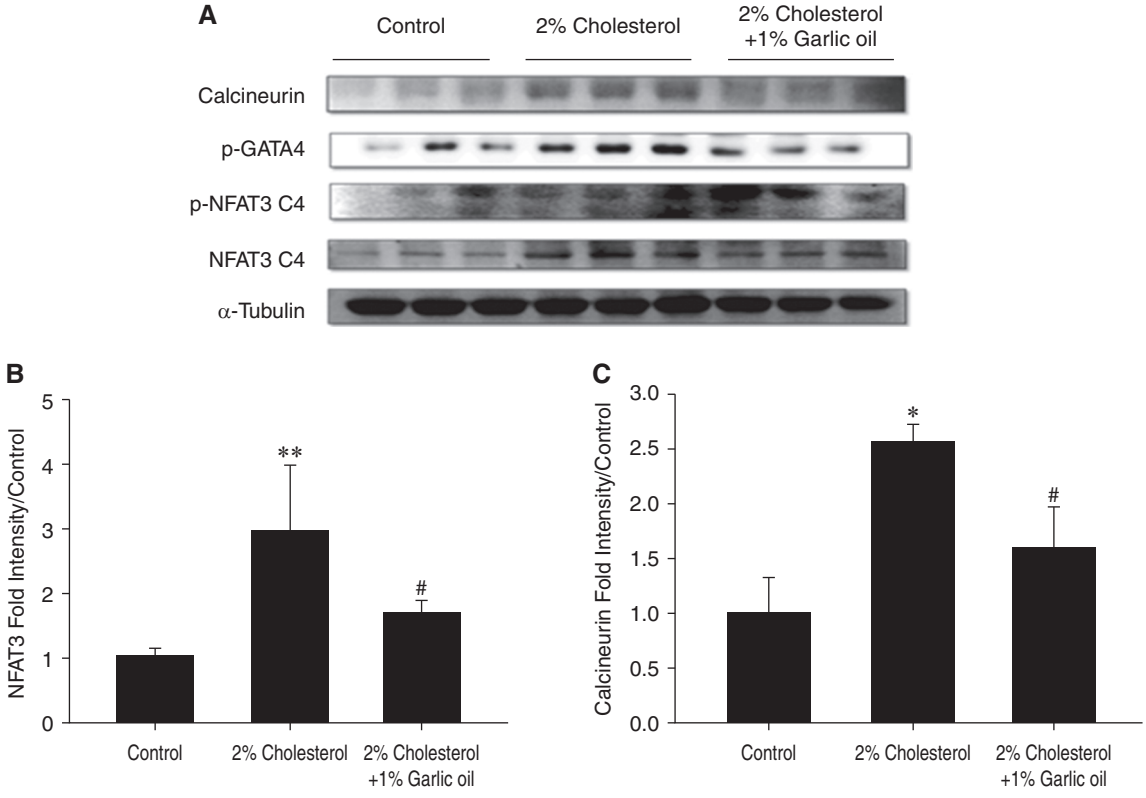

Fig. 3. Effects of garlic oil on the expression of hypertrophic transcription factors, calcineurin, NFAT3, p-NFAT3 and p-GATA4. Western blot analysis (A) and relative quantifications and statistical analysis (B) were performed as in Fig. 2; n = 6 in each group.

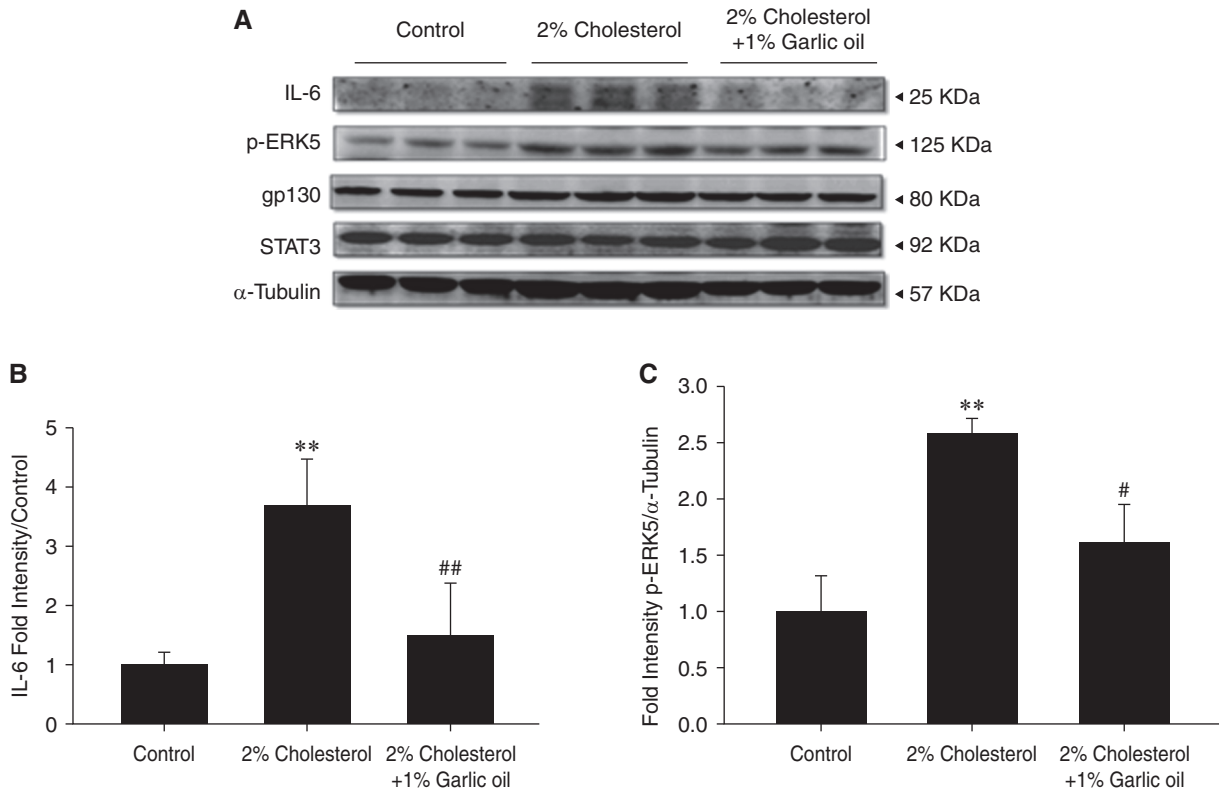

Fig. 4. Effects of garlic oil on the expression of hypertrophy-associated IL-6 signaling pathway proteins, IL-6, STAT3, MEK5 and p-ERK5. Western blot analysis (A) and relative quantifications and statistical analysis (B & C) were performed as in Fig. 2; n = 6 in each group.

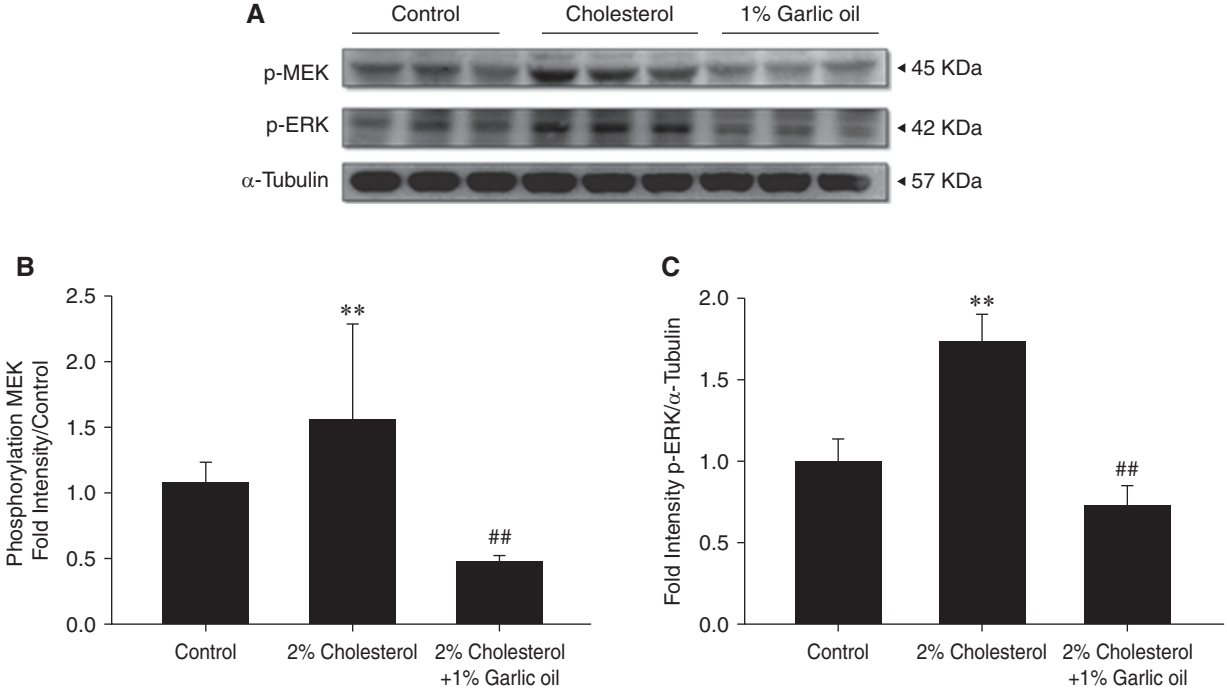

Fig. 5. Effects of garlic oil on the expression of cardiac hypertrophy-associated mitogen-activated protein kinases, ERK and MEK. Western blot analysis (A) and relative quantifications and statistical analysis (B & C) were performed as in Fig. 2; n = 6 in each group.

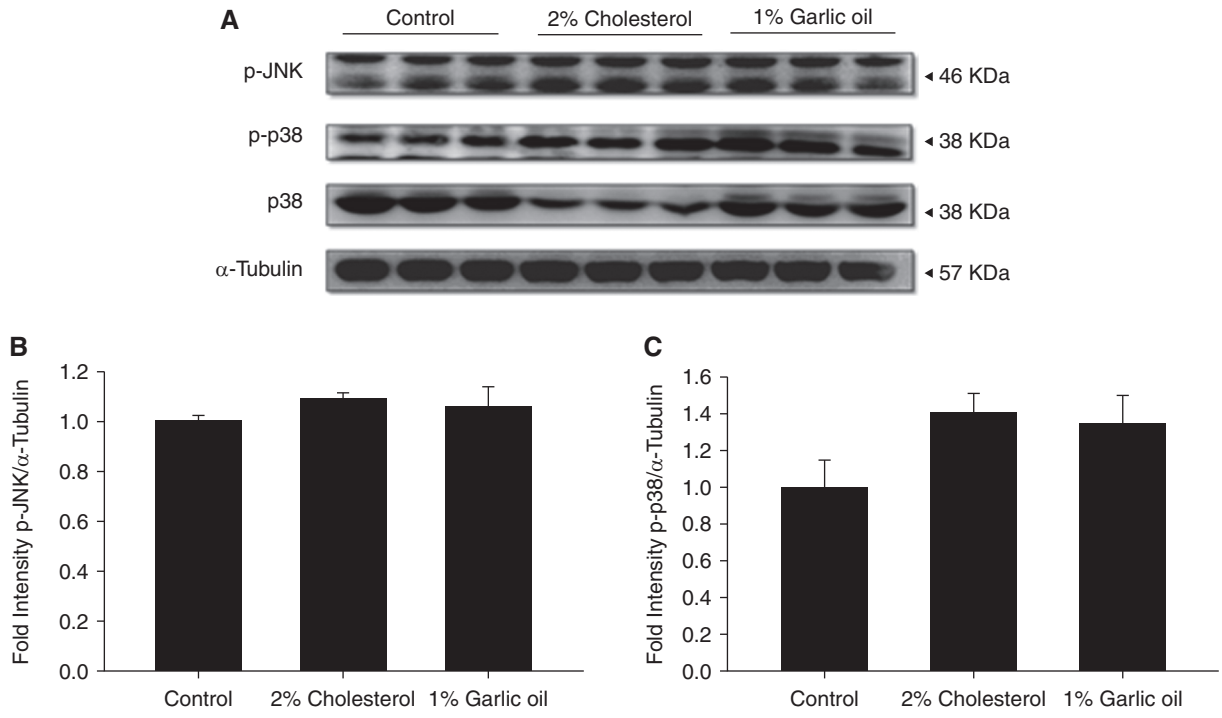

Fig. 6. Effects of garlic oil on the expression of cardiac hypertrophy associated mitogen-activated protein kinases, p38 and JNK. Western blot analysis (A) and relative quantifications and statistical analysis (B & C) were performed as in Fig. 2; n = 6 in each group.

ERK protein was observed in the garlic oil group as compared to the cholesterol group (Fig. 5). However, no significant variations in p-P38 and p-JNK protein levels were detected between the hearts of the control and the cholesterol groups, or between the cholesterol plus garlic oil groups (Fig. 6).

Discussion

Hypercholesterol diets have been postulated as the major sources to cause cardiac hypertrophy that is associated with heart diseases (7, 15, 32, 41). Due to the side effects caused by chemotherapy, there is a worldwide reliance on traditional medicine for therapeutic needs (26). In the current study, we intended to elucidate the effects of garlic oil supplements on cardiac hypertrophy induced by a high cholesterol diet. Our experimental results indicated significant reduction of the WHW/BW and LVW/BW ratios by garlic oil supplements in cholesterol feeding hamsters. The protein levels of ANP, BNP, and hypertrophy-related factors IL-6, STAT3, p-ERK 5, calcineurin, NFAT3, p-GATA4, p-MEK and p-ERK were significantly increased in the cholesterol group whereas significant reduction of these proteins was observed in the garlic oil group.

The alleviating effects of garlic oil on hypercholesterol-related cardiac hypertrophy can potentially reduce the plasma cholesterol levels, and prevent fat-induced hyperlipidemia as garlic is known to cause hypolipidemia and inhibition of atherogenesis (1, 9, 10, 17). Hyperlipidemia is known to increase serum levels of the IL-6. However, the link between hyperlipidemia, apoptosis and inflammation remains largely unknown (8).

IL-6 is a pleiotropic factor associated with various cardiac diseases (20, 31, 39). Elevated IL-6 mRNA is observed in hypertrophic cardiomyopathy patients (39), controling various signaling pathways including the p38 MAPK, STAT1-STAT3 heterodimer pathway, the STAT3 homodimer pathway and the ERK pathway (2, 22, 28, 29, 33, 38). In our experimental results, significant elevated levels of IL-6 expression, as well as hypertrophic-related signaling molecules including p-MEK5 and p-ERK5, were detected in the excised ventricle of hamsters from the cholesterol group, consistent with previous reports (2, 20, 22, 28, 29, 31, 33, 38, 39).

There are no significant differences in the protein levels of gp130 and STAT3 were detected between hamsters from the cholesterol and the garlic oil groups, but significant reductions were detected in the excised ventricle of hamsters from the garlic oil group. To further clarify the signaling pathway involved, we further examined the MAPK pathway which is important in cardiac hypertrophy and which consists of three

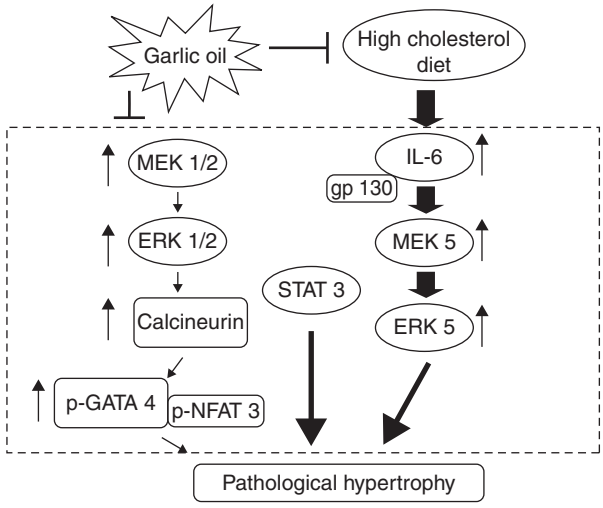

Fig. 7. Schematic presentation of a hypothesis integrating findings of the present study of garlic oil inhibiting cardiac hypertrophy induced by hypercholesterol diet in a hamster model. Cardiac hypertrophy caused by high cholesterol diet also induces pathological hypertrophy, which is mediated by the IL-6-related MEK5–ERK5, STAT3 pathway and other pathways associated with increased phosphorylated MEK1/2 and ERK1/2, signalling pathway. These signaling cascades attenuate calcineurin activity and permit to translocate in to the nucleus. The dephosphorylated NFAT-3 further interacts with GATA-4 transcription factor to form the complex that participates in the development of myocardial hypertrophy and the gene expressions. However, garlic oil administration totally blocked the cardiac hypertrophy effects induced by high cholesterol diet in this hamster model. Dotted line box- molecular events associated with pathological hypertrophy, thick downward arrows- mechanism of IL-6 mediated pathological hypertrophy induced by high cholesterol diet, thin downward arrows- mechanism of MEK1/2 mediated pathological hypertrophy induced by high cholesterol diet, thin upward arrows- trend of respective expression that lead hypertrophy.

major cascades, ERK, JNK and p38 (12, 33). As revealed in the current study, significant increases of p-MEK and downstream p-ERK were observed in the ventricle of hamsters from the cholesterol group. On the other hand, p-MEK and p-ERK levels were significantly reduced in the ventricle of hamsters from the garlic oil group. However, no significant differences in protein levels of p-p38 and p-JNK were detected among the control, cholesterol and the garlic oil groups. These findings suggest that garlic oil acts against cardiac hypertrophy induced by high cholesterol diet *via* attenuation of the p-ERK cascade (Fig. 7).

Garlic oil has been reported to have a variety of cardio vascular effects, including reduction in plasma cholesterol and prevention of fat-induced hyperlipidemia. To elucidate the effect and possible mechanism of garlic oil on hypercholesterolemia-induced cardiac hypertrophy, we performed histopathological analysis and western blotting to investigate changes in the myocardial architecture, and expression of different cardiac hypertrophy-associated molecules in the ventricle of the experimental hamsters. Notably, significant reduced ratios of WHW/BW and LVW/BW were observed in hamsters from the garlic oil group as compared to those from the cholesterol group which suggest the protective effects on cardiac hypertrophy of garlic oil.

Besides the attenuated expression of ANP and BNP, the effects of garlic oil against cardiac hypertrophy are probably exerted *via* the down-regulation of members of the IL-6 receptor pathway and the ERK signaling cascade but not through the JNK and P38 cascades. Taken together, our findings using a hypercholesterol-induced hamster model revealed that garlic oil has significant protective effects against cardiac hypertrophy. Since cardiac hypertrophy effects were restored by the garlic oil feeding, we strongly suggest garlic oil may be useful in the treatment of hypertrophy-associated cardiovascular diseases.

Acknowledgments

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