Abstract. The effects of Sanggenon C on oxidative stress and inflammation have previously been reported; however, little is currently known regarding the effects of Sanggenon C on cardiac hypertrophy and fibrosis. In the present study, aortic banding (AB) was performed on mice to induce cardiac hypertrophy. After 1 week AB surgery, mice were treated daily with 10 or 20 mg/kg Sanggenon C for 3 weeks. Subsequently, cardiac function was detected using echocardiography and catheter-based measurements of hemodynamic parameters. In addition, the extent of cardiac hypertrophy was evaluated by pathological staining and molecular analysis of heart tissue in each group. After 4 weeks of AB, vehicle-treated mice exhibited cardiac hypertrophy, fibrosis, and deteriorated systolic and diastolic function, whereas treatment with 10 and 20 mg/kg Sanggenon C treatment ameliorated these alterations, as evidenced by attenuated cardiac hypertrophy and fibrosis, and preserved cardiac function. Furthermore, AB-induced activation of calcineurin and nuclear factor of activated T cells 2 (NFAT2) was reduced following Sanggenon C treatment. These results suggest that Sanggenon C may exert protective effects against cardiac hypertrophy and fibrosis via suppression of the calcineurin/NFAT2 pathway.

Introduction

Pathological cardiac hypertrophy, which is induced by prolonged and abnormal hemodynamic stress, including hypertension and myocardial infarction, is associated with cardiac fibrosis, capillary rarefaction, increased production of proinflammatory cytokines, cellular dysfunction and undesirable epigenetic alterations. These complex responses lead to maladaptive cardiac remodeling and heart failure (1,2). Numerous signaling cascades/proteins are involved in pathological cardiac hypertrophy, including insulin-like growth factor-phosphoinositide 3-kinase-Akt, mitogen-activated protein kinase pathways, nuclear factor (NF)-κB and calcium signaling (3). Among these, calcium is central to the control of contractile function and cardiac growth (3,4). The best described calcium-dependent signaling proteins include calcineurin and calcium/calmodulin-dependent protein kinases (4). Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) transcription factors, which promotes nuclear translocation and activation of hypertrophic gene transcription (5). Therefore, it is important to identify novel pharmacological agents that target these relevant signaling pathways and improve the long-term clinical outcomes of patients with heart failure.

Sanggenon C is a flavanone Diels-Alder adduct compound, which is isolated from the root bark of Morus cathayana (6). A previous study reported that Sanggenon C may inhibit proteasome function, resulting in the inhibition of tumor cell growth (6). In addition, it has previously been reported that Sanggenon C inhibits inducible nitric oxide synthase expression in RAW264.7 cells (7), and tumor necrosis factor-α-stimulated cell adhesion and vascular cell adhesion molecule-1 expression, by suppressing NF-κB activity (8). Since Sanggenon C possesses antioxidant and anti-inflammatory activities, which serve a key role in cardiac hypertrophy, it may serve as a potential antihypertrophic agent. The present study aimed to determine the effects of Sanggenon C on cardiac hypertrophy.

Materials and methods

Materials. The following primary antibodies were used: Anti-calcineurin A (ab90540; Abcam, Cambridge, MA, USA), anti-phosphorylated (p)-NFAT2 (sc-32994; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-total NFAT2 (ab2796; Abcam), anti-GAPDH (MB001; Bioworld Technology, Inc., St. Louis Park, MN, USA) and anti-LaminB (12255; Cell Signaling Technology, Inc., Danvers, MA, USA). The following secondary antibodies were used: Goat anti-rabbit IRdye® 800CW immunoglobulin G (IgG) (926-3221; LI-COR Biosciences, Lincoln, NE, USA) and goat anti-mouse IRdye® 800CW IgG (926-32210; LI-COR Biosciences). Sanggenon C (98% purity, as determined by
high-performance liquid chromatography analysis) was obtained from Shanghai Medical Technology Development Co., Ltd. (Shanghai, China).

Animals and animal models. A total of 60 male C57/BL6 mice (weight, 23.5-27.5 g; age, 8 weeks, purchased from Beijing HuaFuKang Biological Technology Co, Beijing, China) were housed in an environment with controlled temperature (18-22°C) and humidity (50-60%), under a 12-h light-dark cycle with free access to food and water. The mice were allowed to acclimate for ≥1 week prior to experimentation. All animal protocols were approved by the Animal Care and Use Committees at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China), and all approved animal studies were performed in accordance with the Guide for the Care of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23; revised 1996). Aortic banding (AB) was performed to induce cardiac hypertrophy. Briefly, mice were anesthetized by intraperitoneal injection with 3% sodium pentobarbital (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Artificial respiration was maintained using a rodent ventilator (model, ALC-V8S; Shanghai Alloc Biotech Co., Ltd., Shanghai, China). An incision was made in the second and third intercostal muscles, after which the aortic arch branch was exposed using a chest expander. The vessel was ligated using a 26/27G syringe needle placed parallel above the vessel. In the sham operation group, mice received the same surgery except the last step (the line was not ligated). At the beginning of the AB model, Doppler analysis was performed to ensure that adequate constriction of the aorta and similar pressure overload had been achieved in all groups of AB-operated mice, and pressure gradients (mmHg) were calculated from the peak blood velocity (Vmax, m/sec, PG=4xVmax²). Therefore, 26/27G syringe needles were chosen to ensure 70% aortic coartation of all mice. Following rapid withdrawal of the needle to achieve aortic constriction, the chest was closed in layers and a total of 0.1 ml 0.5% bupivacaine (Sigma-Aldrich; Merck KGaA) was subcutaneously injected close to the edges of the skin incision to alleviate postoperative pain. Mice were divided into four groups: Sham group (control group, n=15, the mice were received sham surgery, and were injected intraperitoneally (0.1 ml/100 g body weight) with saline with 1% Tween-80); Low-dose AB group [n=15, after 1 week of AB, the mice were injected intraperitoneally (0.1 ml/100 g body weight) with saline with 1% Tween-80]; LD-AB group [n=15, after 1 week of AB, the mice were injected intraperitoneally (0.1 ml/100 g body weight) with Sanggenon C (20 mg/kg/day)] for 3 weeks. Four weeks after the operation, the hearts, lungs (0.1 ml/100 g body weight) with Sanggenon C (20 mg/kg/day) were dissected and weighed or measured to compare the heart weight (HW)/body weight (BW) (mg/g), HW/tibia length (TL) (mg/mm), and lung weight (LW)/BW (mg/g) ratios in each group.

Echocardiography and hemodynamics. A total of 4 weeks after surgery, mice were subjected to echocardiographic analyses and hemodynamic measurements. Echocardiography was performed using MyLab 30CV (Esaote SpA, Florence, Italy) with a 10 MHz linear array ultrasound transducer. Dimensions of the left ventricle (LV) were assessed in parasternal long-axis and short-axis views at a frame rate of 50 Hz. End-systole or end-diastole was defined as the phase in which the LV area was smallest or largest, respectively.

For hemodynamic measurements, mice were anesthetized with 1.5% isoflurane, and a microtip catheter transducer (SIR-839; Millar, Inc., Houston, TX, USA) was inserted into the LV via the right carotid artery. The signals were recorded using a Millar Pressure-Volume system (MPVS-400; Millar, Inc.), and cardiac output (CO), end-diastolic pressure (EDP), end-systolic pressure (ESP), maximal rate of pressure development (dP/dt max) and minimal rate of pressure decay (dP/dt min) were analyzed using the PVAN data analysis software version 2.3 (Millar, Inc.).

Histological analysis. After mice were sacrificed, mice hearts were removed, arrested in diastole with 10% KCl, weighed, fixed by perfusion with 10% formalin, and embedded in paraffin. Hearts were transversely cut close to the apex to visualize the left and right ventricles. Several heart sections (4-5 μm) were prepared, stained with hematoxylin and eosin for histopathology for 5 min at room temperature or with Picro Sirius Red for 90 min at room temperature to detect collagen deposition, and were then visualized by light microscopy. A single myocyte was measured using an image quantitative digital analysis system (Image Pro-Plus, version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The outline of 100 myocytes was traced in each group.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was used to detect the mRNA expression levels of hypertrophic and fibrotic markers. Total RNA was extracted from frozen, pulverized mouse cardiac tissue using TRIzol (15596026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA yield and purity were spectrophoto metrically estimated using A260/A280 and A230/A260 ratios via a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RNA (2 μg/sample) was reverse-transcribed into cDNA using oligo (DT) primers and the Transcriptor First Strand cDNA Synthesis kit (04896866001; Roche Diagnostics Corporation, Indianapolis, IN, USA), according to the manufacturer’s protocol. qPCR was conducted using a LightCycler 480 SYBR-Green I Master mix (04707516001; Roche Diagnostics Corporation, Indianapolis, IN, USA), according to the manufacturer’s protocol. qPCR was conducted using a LightCycler 480 SYBR-Green I Master mix (04707516001; Roche Diagnostics Corporation). Briefly, after a 5 min initial denaturation at 95°C, a total of 42 primer-extension cycles were performed. Each cycle consisted of a 10 sec denaturation step at 95°C, a 20 sec annealing step at 60°C, a 1.5 sec extension step at 72°C for extension and a final extension step at 72°C for 10 min. Analysis of relative gene expression was performed using the 2^ΔΔCq method (9). The results were normalized to the mRNA expression of GAPDH. The oligonucleotide primers are demonstrated in Table I.

Western blotting. Cardiac tissues were lysed in radioimmuno precipitation assay lysis buffer (RIPA), containing 720 μl RIPA, 20 μl PMSF (1 mM), 100 μl complete, 100 μl complete (04693124001; Roche, Indianapolis, IN, USA), 100 μl photostop (04906837001; Roche), 50 μl NaF (1 mM) and 10 μl NaVO₄. Protein concentration was measured using a Bicinchonic
Table I. Primer sequences for RT-PCR assays.

| mRNA   | Forward (5'-3')                  | Reverse (5'-3')                  |
|--------|----------------------------------|----------------------------------|
| ANP    | ACCTGCTAGACCCACCTGGAG            | CTTTGCGTGTATCTTTCGGTACCACG       |
| BNP    | GAGGTCACTCTCTATCTCTCTGG          | GCCATTTCCTCCGACATTITCTC          |
| b-MHC  | CCGAGTCCAGGTCAAACAA             | CTTCAACGGGCAACCTTGGGA           |
| a-MHC  | GTCAAAGTTCCGCAAGGT              | AAGGTTCGTCGGAGAGGTATTA          |
| Collagen I | AGGGTTCTAGGTGGTTAGT           | CACCAACAGCCACATCCGTAA          |
| Collagen III | CCAACCAGGAGATTCCATT           | GAAGCACAGGAGCAGTGTAGA          |
| Fibronectin | CCGTGGTGTGTCAGTCAGA         | GGGTCCCACGTCTGATTTATC         |
| CTGF   | TGGTGATAGGCGCCAAAGGC          | AGTTGGCTCGCATCATAGTTG          |
| GAPDH  | ACTCCACTCAGGGCAATTC            | TCTCCATGGTGGTGAAGACA          |

Acid Protein Assay kit (23227; Pierce; Thermo Fisher Scientific, Inc.) with a Synergy HT ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The protein lysates (50 µg) were separated by 10% SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), which were blocked with 5% non-fat milk at room temperature for 2 h. Following incubation with the primary antibodies (1:200 dilution) overnight at 4°C, the membranes were incubated with goat anti-rabbit or anti-mouse IgG secondary antibodies (1:10,000 dilution). The blots were scanned and analyzed using a two-color infrared imaging system (Odyssey CLx; LI-COR; Biosciences).

H9c2 cardiomyocyte culture. The H9c2 embryonic rat heart-derived cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO₂ at 37°C. Cells were seeded at a density of 5x10³ cells/well in 24-well plates for immunofluorescence detection. Cells were cultured in serum-free medium for 8 h and were pretreated with various concentrations of Sanggenon C (1 and 10 µM) for 12 h prior to phenylephrine (PE; 50 µM; Sigma-Aldrich; Merck KgaA) stimulation for 24 h at 37°C.

Immunofluorescence staining. Briefly, cells were washed with PBS, fixed with RCL2 (Alphelys, Plaisir, France), permeabilized in 0.1% Triton X-100 (Amresco, Solon, OH USA) in PBS, and stained with anti-NFAT2 (ab2796; 1:100; Abcam) in 1% goat serum (Beyotime Institute of Biotechnology, Haimen, China) overnight at 4°C. After washing 5 times with PBS, the cells were then incubated with Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (1:200 dilution; cat. no. A-11011; Invitrogen; Thermo Fisher Scientific, Inc.) for 60 min at room temperature. The cells were washed a further 6 times with PBS, after which, cells on coverslips were mounted onto glass slides using SlowFade Gold Antifade Mountant with DAPI (S36939; Invitrogen; Thermo Fisher Scientific, Inc.). The cells were visualized by light microscopy (model BX51TRF; Olympus Corporation, Tokyo, Japan).

Statistical analysis. Values are expressed as the mean ± standard error of the mean. All experiments were repeated independently 3 times. Data were analyzed using a one-way analysis of variance followed by a post hoc Tukey test. Comparisons between two groups were performed by unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were conducted using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Sanggenon C improves impaired cardiac function following AB. In order to determine whether Sanggenon C protects the heart from pressure overload-induced cardiac dysfunction, mice were subjected to AB surgery or sham surgery. Echocardiography measurements in the vehicle-AB group exhibited significantly increased chamber diameter, and decreased LV ejection fraction (LVEF) and fractional shortening (LVFS). Treatment with 10 mg/kg (low dosage, LD) and 20 mg/kg (high dosage, HD) Sanggenon C prevented the development of ventricular dysfunction, as evidenced by decreased LV end-diastolic diameter, LV end-systolic diameter, and increased LVFS and LVEF (Fig. 1A). Hemodynamic measurements in the vehicle-AB group exhibited significantly increased diastolic blood pressure, and decreased systolic and diastolic function; however, mice treated with HD Sanggenon C demonstrated normalization of these hemodynamic parameters, including EDP, CO, dP/dt max and dP/dt min (Fig. 1B). Treatment with LD Sanggenon C only decreased EDP after 4 weeks of AB (Fig. 1B).

Sanggenon C protects against cardiac hypertrophy. A total of 4 weeks following AB surgery, mice in the vehicle-AB group exhibited obvious cardiac hypertrophy as evidenced by increased cardiac mass, myocyte cross sectional area (CSA), HW/BW, HW/L and LW/L, compared with in the sham group. LD and HD Sanggenon C-treated mice exhibited attenuated cardiac hypertrophy compared with vehicle-AB mice, as indicated by decreased CSA (Fig. 2A), and reduced HW/BW and HW/L ratios. In addition, the development of pulmonary congestion (LW/L ratio), which as an indirect indicator of heart failure, was significantly decreased in the LD and HD Sanggenon C-treated mice (Fig. 2B). The
hypertrophic markers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (MHC) were markedly reduced, whereas α-MHC was increased, in LD and HD Sanggenon C-treated mice in response to AB.
In addition, treatment with HD Sanggenon C exerted better protection against hypertrophy. These results suggested that Sanggenon C may negatively regulate the extent of cardiac hypertrophy and preserve cardiac function in response to pressure overload.

**Sanggenon C attenuates pressure overload-induced cardiac fibrosis.** To determine the extent of fibrosis in the heart, paraffin-embedded slides were stained with PSR. Perivascular and interstitial fibrosis was detected in each group. Marked interstitial and perivascular fibrosis was observed in mice in

(Fig. 2C). In addition, treatment with HD Sanggenon C exerted better protection against hypertrophy. These results suggested that Sanggenon C may negatively regulate the extent of cardiac hypertrophy and preserve cardiac function in response to pressure overload.
the vehicle-AB group, which was attenuated following treatment with LD and HD Sanggenon C (Fig. 3A). Subsequently, the mRNA expression levels of known mediators of fibrosis were detected, including collagen Ia, collagen III, fibronectin...
and connective tissue growth factor (CTGF), the results demonstrated a marked reduction in expression in LD and HD Sanggenon C-treated mice (Fig. 3B). In addition, treatment with HD Sanggenon C exerted better protective effects against cardiac fibrosis. However, treatment with HD Sanggenon C alone did not affect the mRNA expression levels of hypertrophic and fibrotic markers compared with the vehicle-sham group (Fig. 3C).

Sanggenon C inhibits the calcinuerin/NFAT2 signaling pathway in response to hypertrophic stimuli. To determine the mechanism underlying the antihypertrophic actions of
Sanggenon C, the calcineurin/NFAT2 signaling pathway, which is well recognized to be involved in the development of cardiac hypertrophy, was assessed by western blotting. The results demonstrated that AB-induced upregulation of calcineurin A in the cytoplasm, and nuclear translocation of NFAT2 in the heart, was significantly suppressed following treatment with LD and HD Sanggenon C. In addition, AB-mediated reductions in the cytoplasmic expression of p-NFAT2 and total NFAT2 were significantly ameliorated in LD and HD Sanggenon C-treated mice hearts compared with in vehicle-treated mice hearts (Fig. 4A and B). To further confirm these results in vitro, H9c2 rat cardiomyocytes were stimulated with PE (50 µM) and were treated with various concentrations of Sanggenon C (1 and 10 µM). Immunofluorescence staining of NFAT2 indicated that Sanggenon C inhibited the nuclear translocation of NFAT2 in cardiomyocytes (Fig. 4C). Based on these findings, it may be suggested that Sanggenon C regulates cardiac remodeling, at least in part, via the calcineurin A/NFAT2 pathway.

Discussion

Sanggenon C, which is isolated from the root bark of Morus cathayana, is a natural prenylated flavanone Diels–Alder adduct extracted from the Chinese crude drug Shangbaipi (10). At present, there are only a few reports regarding the biological and pharmacological effects of Sanggenon C. Much of the recent work has focused on the antioxidative (11), anti-inflammatory (7) and antitumor (6) activities of Sanggenon C. The present study used AB to induce a cardiac hypertrophic model in mice. The results demonstrated that Sanggenon C treatment significantly attenuated cardiac hypertrophy and fibrosis under conditions of pressure overload, and cardiac function was protected. These results suggested that Sanggenon C exerts antihypertrophic activities.

The mechanism by which Sanggenon C mediates its antihypertrophic effect remains unclear. Previous studies have demonstrated that various signal transduction pathways and transcription factors are involved in the development of cardiac hypertrophy (12,13). Calcineurin is an important signal transduction factor that regulates growth and stress responses in various cell types in the progression of cardiac remodeling to heart failure (14). An important downstream target of calcineurin is the family of NFAT1–4 transcription factors, which was originally described as a transcriptional activator of cytokines and immunoregulatory genes in T cells. These transcription factors, each of which is expressed in the myocardium, are initially phosphorylated and cytosolic under basal conditions, but are able to translocate to the nucleus upon stimulation and dephosphorylation by calcineurin, subsequently resulting in activation of pathological hypertrophic gene expression (15,16). Pharmacological studies support that calcineurin-inhibiting agents, including cyclosporine A and FK506, can attenuate agonist-induced cardiac hypertrophy (17,18). However, when considering the use of these calcineurin inhibitory agents as therapeutics for the treatment of human heart disease, the drug side effects should be taken into consideration, since cyclosporine A and FK506 may inhibit additional targets. Therefore, other pharmacological agents may be more desirable to further decipher the role of the calcineurin/NFAT signaling pathway in cardiac remodeling. The present study examined the effects of Sanggenon C on hypertrophic pathways. The results demonstrated that calcineurin activation and NFAT2 nuclear translocation were attenuated in Sanggenon C-treated mice. Chronic cardiac pressure overload induces adrenal medulla hypertrophy and increased catecholamine synthesis, which activates the G-protein coupled receptor signaling pathway (19). The present study used PE to induce cardiac hypertrophy in cardiomyocytes in vitro, and NFAT2 nuclear translocation was reduced by Sanggenon C treatment. Therefore, it may be hypothesized that the protective role of Sanggenon C on cardiac hypertrophy is dependent on suppression of the calcineurin/NFAT signaling pathway.

Pathological cardiac hypertrophy involves excessive deposition of extracellular matrix (ECM) proteins by cardiac fibroblasts, which reduces tissue compliance and accelerates the progression of heart failure (20,21). A complex interaction among a network of growth factors/cytokines and hormones is responsible for the activation of fibroblasts, promotes persistence of myofibroblasts and induces the expression of various ECM components, including collagen I, collagen III and fibronectin (22,23). As suggested in the present study, Sanggenon C was able to negatively regulate CTGF, collagen I, collagen III and fibronectin production in response to pressure overload. However, the mechanism underlying Sanggenon C-mediated anti-fibrotic effects remains unclear. Further studies are required to confirm whether Sanggenon C mediates its antifibrotic effect directly via fibroblasts, or whether it indirectly regulates cardiac fibrosis by protecting against cardiac hypertrophy.

In conclusion, the present study indicated that Sanggenon C may protect against cardiac hypertrophy and fibrosis in response to chronic pressure overload via inhibition of the calcineurin/NFAT signaling pathway. Therefore, Sanggenon C may be considered a novel therapeutic agent for the treatment of human heart disease.

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