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Original Article

Gambogic acid suppresses pressure overload cardiac hypertrophy in rats

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Abstract: Cardiac hypertrophy is a common response of the heart to a variety of cardiovascular stimuli. Pathological cardiac hypertrophy eventually leads to heart failure. Gambogic acid (GA) is a main active ingredient isolated from the gamboge resin of *Garcinia hanburyi* trees and has potent anti-tumor and anti-inflammatory effects that are associated with inhibition of the NF-κB pathway. We and others recently reported that GA can significantly inhibit the function of the proteasome with much less toxicity than conventional proteasome inhibitors. The increasing lines of evidence indicate that the inhibition of the proteasome can promote the regression of cardiac hypertrophy induced by pressure overload through the blockade of the NF-κB pathway. In the present study, we examined the effect of GA on pressure overload or isoproterenol infusion induced cardiac hypertrophy and fibrosis, and changes in myocardial NF-κB signaling. We observed that the heart weight/body weight ratio, the size of cardiomyocytes, interstitial fibrosis, and the reactivation of fetal genes (α-SK-actin and BNP mRNA) were markedly increased by abdominal aorta constriction (AAC) or isoproterenol infusion (ISO), all of which were effectively inhibited by GA treatment. Furthermore, GA treatment abolished proteasome chymotrypsin-like activity increases induced by AAC or ISO, led to increased myocardial IκB protein, decreased NF-κB p65 subunit levels in the nuclear fraction, decreased NF-κB DNA-binding activity, and reduced IL2 levels in the myocardium of rats subject to AAC or ISO. In conclusion, GA treatment can suppress cardiac hypertrophy and fibrosis induced by pressure overload or isoproterenol possibly through the inhibition of the proteasome and the NF-κB pathway, suggesting that GA treatment may provide a new strategy to treat cardiac hypertrophy.

Keywords: Gambogic acid, cardiac hypertrophy, pressure overload, isoproterenol, proteasome, NF-κB

Introduction

In adult mammalian hearts, cardiomyocytes possess very poor, if any, proliferation capability. Consequently, the heart responds to increased workload or stress by increasing the size, instead of the number, of cardiomyocytes. The increase in cardiomyocyte size is referred to as cardiac hypertrophy. Initially, cardiac hypertrophy is usually an important adaptive response to many forms of cardiovascular stress, such as hypertension, valvular heart disease, and myocardial infarction; however, the hypertrophy becomes maladaptive when the stress sustains and the hypertrophic heart eventually transitions to heart failure [1]. In the process of cardiac hypertrophy, protein synthesis and degradation are both increased with the increase of synthesis apparently surpassing the degradation. Like in other cells, protein degradation in cardiomyocytes is mainly carried out by the ubiquitin-proteasome system (UPS) [2, 3]. The UPS degrades a target protein generally through two main steps: the covalent attachment of a chain of ubiquitin molecules to the substrate protein via a process known as ubiquitination and subsequently the degradation of the polyubiquitinated protein individually by the protea-

some [4]. Accumulating evidence has shown that the functional alteration of the UPS can be involved in the development of cardiac hypertrophy, especially the increase of proteasome expression and activity in myocardium shown in response to pressure overload [5, 6]. Therefore, the inhibition of the proteasome may theoretically suppress cardiac hypertrophy induced by pressure overload. Indeed, several studies have tested this hypothesis using proteasome inhibitors such as epoxomicin, MG132 (Z-Leu-Leu-Leu-al), bortezomib, and PS519 [7-10]. Although some of these studies have shown the proteasome inhibitors to be antihypertrophic, the differential susceptibility of cardiac proteasome subtypes to proteasome inhibitors, and cardiac toxicity will affect their use as antihypertrophic agents [11]. Accordingly, more effective drugs with no or lower toxicity to suppress cardiac hypertrophy are still urgently needed.

Gambogic acid (GA) is one of the main active ingredients isolated from the gamboge resin of *Garcinia hanburyi* tree [12]. In addition to being used as a type of dye for textile staining, the gamboge resin has been used in traditional Chinese medicine to promote detoxification and hemostasis and as an agent to kill parasites. The vast majority of studies have demonstrated that GA and its derivatives have potent anti-tumor and anti-inflammatory effects [13-16]. Our previous study has shown that GA is a more tissue-specific proteasome inhibitor with much less toxicity compared to bortezomib [17]. The proteasome inhibition effect of GA was subsequently demonstrated also by Felth J and colleague [18]. More recently, we reported that GA could efficiently attenuate right ventricular hypertrophic responses induced by hypoxia [14]. However, it remains to be evaluated whether GA, as an effective proteasome inhibitor, could attenuate cardiac hypertrophic responses induced by pressure overload.

A few studies have suggested that the activation of the NF-κB pathway plays an important role in the development of cardiac hypertrophy, and thus the inhibition of NF-κB pathway could promote the regression of cardiac hypertrophy induced by pressure overload [19, 20]. The activation of NF-κB requires proteasome-mediated degradation of IκB [21, 22]. Hence, as a tissue-specific proteasome inhibitor, GA may block the NF-κB pathway via inhibiting the degradation of IκB in its target tissues. Moreover, it has been shown recently that GA could inhibit the NF-κB activation through directly targeting the upstream molecule of the NF-κB pathway [14]. Therefore, we tested in the present study the hypothesis that GA could suppress cardiac hypertrophy induced by pressure overload through the inhibition of the proteasome and the NF-κB pathway. Here, we report that GA can effectively suppress the elevation of myocardial proteasome peptidase activity, the activation of the NFκB pathway in myocardium, as well as cardiac hypertrophic responses induced by abdominal aorta constriction (AAC) or isoproterenol infusion (ISO) in rats.

Materials and methods

Animals

The protocol for the care and use of all animals in this study was approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University (Guangzhou, China). Male Sprague-Dawley rats at 6 weeks of age were obtained from Guangdong Laboratory Animal Monitoring Institute. All animals had access to standard laboratory diet and drinking water *ad libitum*.

Induction of left ventricular hypertrophy via abdominal aorta constriction (AAC)

The animal models were created as described previously [7]. All animals were randomly divided into three groups (6 rats per group): vehicle-treated sham group (sham group), vehicle-treated AAC group (AAC group) and GA-treated AAC group (AAC+GA group). Briefly, the animal was anesthetized via intraperitoneal injection of 2% sodium pentobarbital (2 ml/kg) and the abdominal aorta was exposed by a midline abdominal incision. Silk sutures (5-0) were used to ligate the aorta against 22-gauge blunted needle, and then the needle was rapidly retracted to create a defined constriction. For the sham group, rats underwent the same surgery without aortic constriction. At day 2 after the surgery, rats of the AAC+GA group were administered via intraperitoneal injection of 1.5 mg/kg GA (Enzo, USA) once every other day for 2 weeks. Rats of the sham group as well as the AAC group received the corresponding volume of vehicle.

Induction of left ventricular hypertrophy by isoproterenol (ISO)

This was performed as described previously [10]. All animals were randomly divided into three groups (6 rats per group): vehicle-treated control group (Control group), vehicle-treated ISO group (ISO group) and GA-treated ISO group (ISO+GA group). The model was induced by administering 30 mg/kg/day ISO (Sigma-Aldrich, USA) subcutaneously for 8 consecutive days. The rats in ISO+GA group were administered via intraperitoneal injection of 1.5 mg/kg GA once every other day for the subsequent 7 days. Rats of other groups were administered with a corresponding volume of vehicle.

Histological analysis

All rats were sacrificed by cervical dislocation at designated times after GA treatment. The cardiac tissue sections were formalin-fixed, paraffin-embedded, and cut into 5-μm serial sections. The sections were then processed for hematoxylin-eosin (HE) and Masson's trichrome staining.

Real-time PCR

The isolation of RNA was performed from cardiac samples using TRIzol reagent (Invitrogen, USA) according to the protocol provided by the manufacturer. The concentration and the purity of RNA were assessed based on the absorbance at 260 nm and the ratio of the absorbance at 260 and 280 nm (A260/280), respectively. PrimeScript® RT Master Mix Perfect Real Time assay kit (Takara, Japan) was used to perform the conversion of RNA to cDNA, and the amplification was performed using an ABI 7000 Sequence Detection System (Applied Biosystems, USA). The target genes for the experiment included α-skeletal-actin (α-SK-actin), brain natriuretic peptide (BNP) and hypoxanthine phosphoribosyltransferase (HPRT). The results were presented as relative expression to HPRT using the $2^{-\triangle\triangle Ct}$ method. Primer sequences were as follows: α-SK-actin-F: 5'-TCA CTT CCT ACC CTC GGC AC-3'; α-SK-actin-R: 5'-AGG CCA GAG CCG TTG TCA CA-3'. BNP-F: 5'-GGT CTC AAG ACA GCC CTT C-3'; BNP-R: 5'-A-CA ACC TCA GCC CGT CAC AG-3'. HPRT-F: 5'-GTA ATG ATC AGT CAA CGG GGG AC-3'; HPRT-R: 5'-CCA GCA AGC TTG CAA CCT TAA CCA-3'.

Proteasomal chymotrypsin (CT)-like peptidase activity assay

The assay was performed according to the protocol we previously used with the synthetic fluorogenic peptide substrate Suc-LLVY-AMC (Calbiochem, USA) [17]. Briefly, cardiac tissues were treated in assay buffer (20 mM Tris-HCl, pH7.5), and then certain concentrations of extracts were co-incubated with Suc-LLVY-AMC for 2.5 hr at 37°C. Luminescence/fluorescence microplate reader (Varioskan Flash 3001, Thermo, USA) was used to detect fluorescence signal.

Western blot analysis

Briefly, equal amounts of total protein extracted from heart tissues were resolved by SDS-PAGE (12% gels), and then transferred to PVDF membranes. The membrane was incubated with primary antibodies at the recommended dilution, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies from appropriate species. Detection was performed by the enhanced chemiluminescence (ECL) system.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay was carried out as described previously [23]. In brief, the nuclear extracts from cardiac tissue were prepared using a Nuclear and Cytoplasmic Protein Extraction Reagent kit (Kaiji, China) according to the manufacturer's protocol. The oligonucleotide probes were generated by end labeling with DIG-dUTP and 0.4 ng/μl labeled probes were produced using DIG Gel Shift kit (Roche, Germany). All other steps were conducted strictly following the manufacturer's instructions.

Statistical analyses

All data in the experiment were presented as mean ± SE. One-way ANOVA was used to determine the differences among groups using SPSS 11.0 software (SPSS Inc., USA), p value <0.05 was considered statistically significant.

Results

Cardiac gravimetric parameters

The body weight (g) and heart weight (mg) of each rat were measured in the terminal experi-

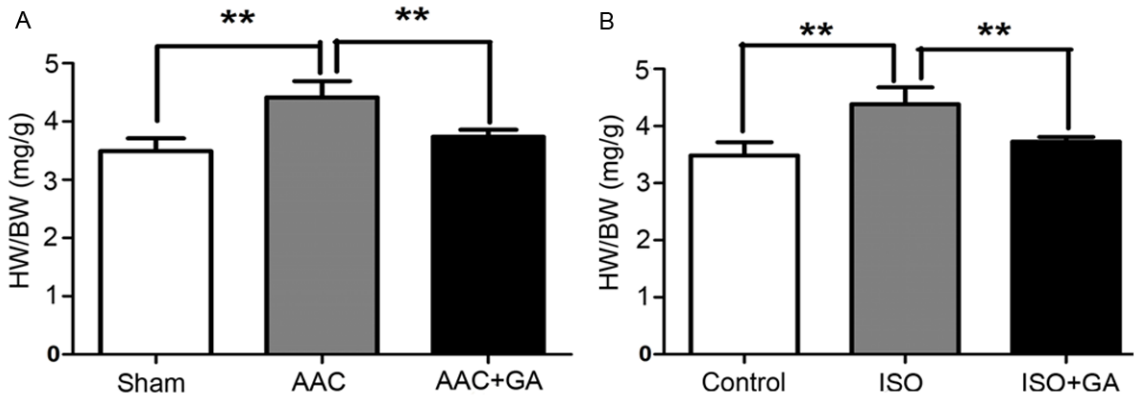

Figure 1. Changes in the heart weight (mg) to body weight (g) ratio (HW/BW) in the terminal experiments. A: Rats were subject to sham surgery (Sham) or aortic abdominal constriction (AAC). Starting day 2 after AAC, the AAC rats were treated with vehicle control (referred to as the AAC group) or gambogic acid (referred to as the AAC+GA group) once every other day for 2 weeks. B: Rats were injected subcutaneously with either vehicle control (Veh) or isoproterenol (ISO, 30 mg/kg/day) for 8 consecutive days. One day after the initiation of ISO injection, the ISO injected rats received vehicle control (the ISO group) or gambogic acid (the ISO+GA group) once every other day for 7 days. Changes in the heart weight (mg) to body weight (g) ratio (HW/BW) in the terminal experiments were recorded. N = 6 rats/group, $^{**}p<0.01$.

ments. The ratio of heart weight to body weight (HW/BW) in the AAC group was significantly increased as compared to the sham group ($p<0.01$). GA treatment could significantly decrease the ratio of HW/BW ($p<0.01$) (**Figure 1A**). Similarly, HW/BW ratio was significantly increased in the ISO group compared with the Control group ($p<0.01$) but this increase was significantly attenuated by GA treatment ($p<0.01$) (**Figure 1B**). All these findings indicate that GA can suppress cardiac hypertrophy induced by either AAC or ISO infusion.

Histopathological analysis

Increases in cardiomyocyte cross-sectional area and interstitial fibrosis of myocardium are the main characteristics of cardiac hypertrophy and remodeling induced by pressure overload at the histology level. Therefore, histological studies of the myocardium were performed to assess the cardiomyocyte hypertrophy and interstitial fibrosis. In H-E staining, obvious increases in cardiomyocyte cross-sectional area were observed in AAC groups and ISO groups; but this increase was attenuated after administration of GA (**Figure 2A**, **2B**). The Masson's trichrome staining revealed increased interstitial fibrosis in AAC groups and ISO groups; this increase was remarkably smaller in the GA-treated groups (**Figure 2C**, **2D**). These results further indicate that GA is a potent antihypertrophic agent.

Effects of GA on the ventricular myocardial expression of fetal genes in the hypertrophic hearts

Many genes such as α-skeletal actin (α-SK-actin) and BNP, which are expressed in embryonic and fetal hearts, are no longer expressed in the adult heart. The expression of these so-called fetal genes are reactivated when the heart undergoes pathological insults such as pathological hypertrophy [24]. The reactivation of the fetal gene program has been extensively used as an important marker at the gene expression level for cardiac pathological responses [25]. Hence, we extracted total RNA from the ventricular myocardial samples and the RNA was used for measuring the gene expression. Real time PCR was thus performed to assess the effects of GA on the relative expression levels of α-SK-actin and BNP mRNA in the cardiac tissue. Indeed, both AAC and ISO induced increases in the mRNA levels of both α-SK-actin and BNP and the increases were considerably less in AAC+GA (**Figure 3A**, **3B**) or ISO+GA groups (**Figure 3C**, **3D**). These data show that GA can effectively suppress the reactivation of the fetal gene program induced by pressure overload or prolonged and excessive β-adrenergic stimulation.

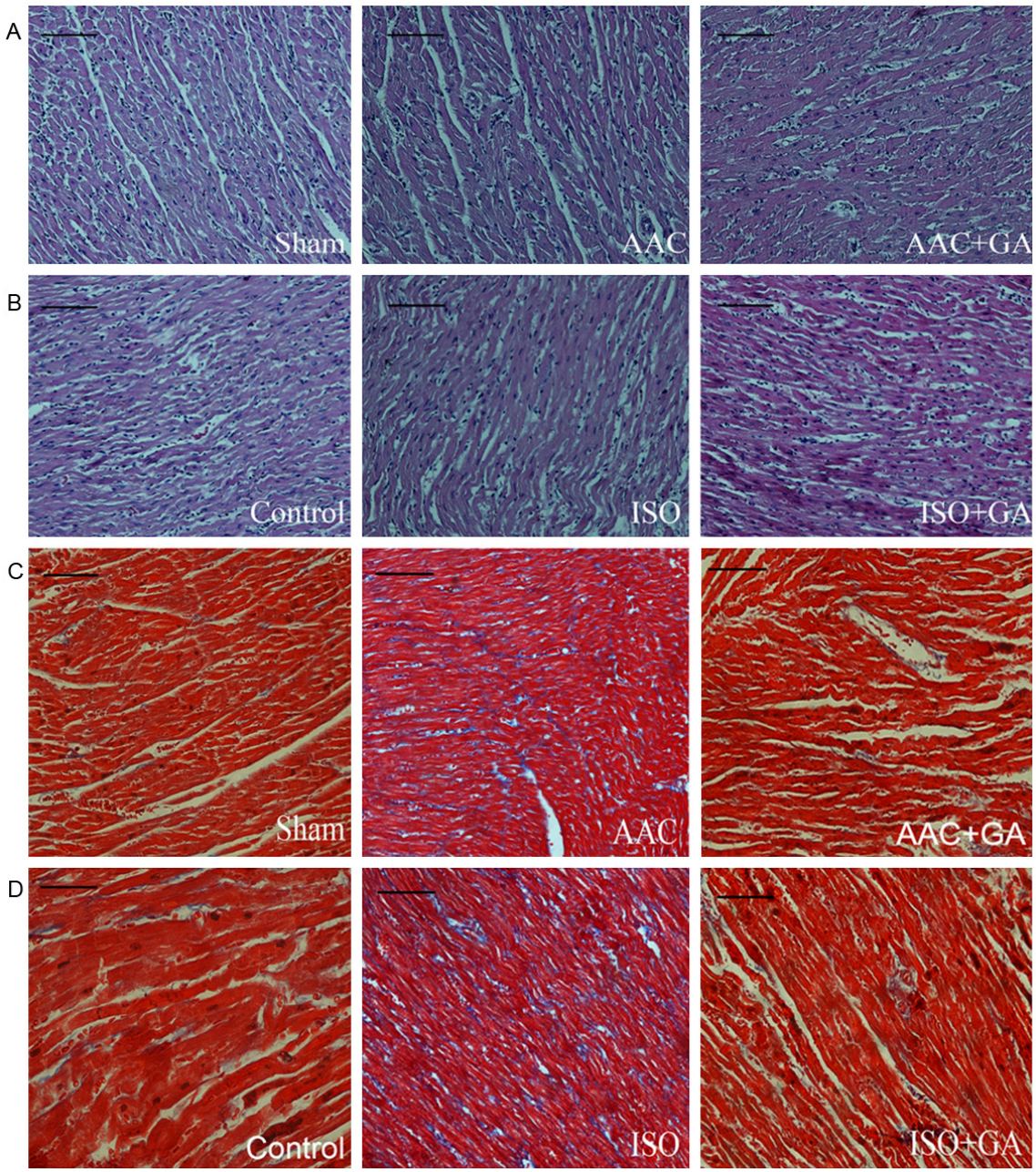

Figure 2. Histopathological assessments. Myocardial tissue samples collected form the indicated groups were fixed and processed to obtain paraffin-embedded tissue sections. A and B: Representative micrographs of H-E stained myocardial sections. Increases of myofiber thickness and myofiber disarray in rats exposed to either abdominal aortic constriction (AAC) or isoproterenol (ISO) were attenuated by gambogic acid (GA) treatment. C and D: Representative micrographs of myocardial sections with Masson's trichrome staining. Areas with blue staining are interstitial fibrotic tissues. Cardiac fibrosis remarkably increased in rats exposed to either AAC or ISO but this increase was considerably reduced by GA treatment. Scale bar = 100 μm.

Effects of GA on the proteasome activity in cardiac tissue

As mentioned earlier, GA metabolites can potently inhibit the proteasome [17]. Hence, we performed the proteasomal chymotrypsin-like (CT-like) peptidase assay to examine the effect of GA on myocardial proteasome activity in the heart undergoing pressure overload or ISO infusion induced cardiac hypertrophy. Compared

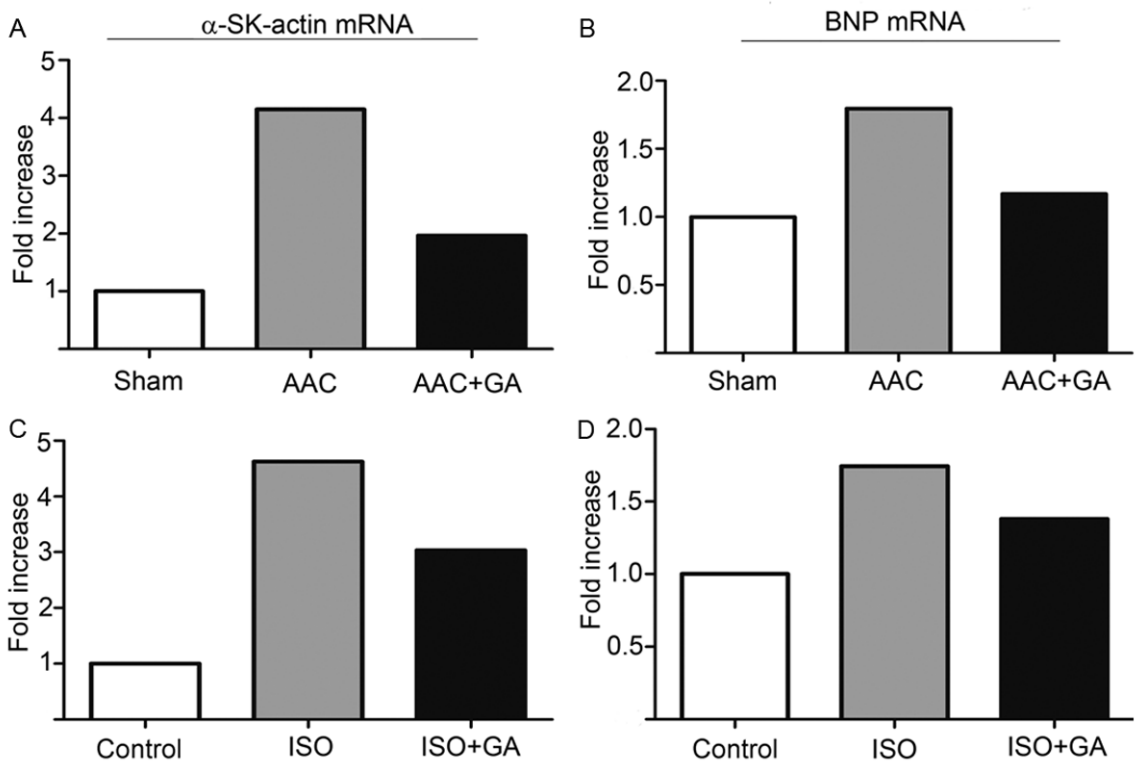

Figure 3. Relative mRNA expression levels of α-SK-actin and BNP in the ventricular myocardium. The relative mRNA expression levels of α-skeletal actin (α-SK-actin) and the brain type of natriuretic peptide (BNP) in the cardiac tissue of rats exposed to AAC (A, B) or ISO (C, D) were measured by using real-time PCR. Results of a representative cohort out of 3 sets are shown. GA, gambogic acid; ISO, isoproterenol infusion.

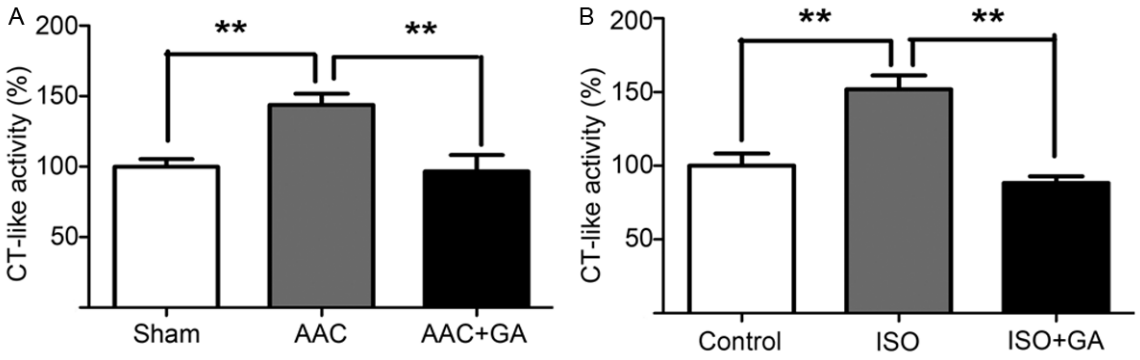

Figure 4. Effects of gambogic acid (GA) treatment on myocardial proteasome chymotrypsin-like (CT-like) activity in rats subject to abdominal aortic constriction (AAC) or isoproterenol (ISO) infusion. Crude myocardial protein extracts from the left ventricles of rats of the pressure overload cohort (A) and the ISO infusion cohort (B) were used for the CT-like assays. The synthetic fluorogenic peptide (Suc-LLVY-AMC) was used as the substrate. N = 6 rats/group; $^{**}p<0.01$.

with the sham controls, myocardial proteasomal CT-like activity was increased by ~50% in the AAC group ($p<0.01$) but this increase was completely abolished in the AAC+GA group ($p<0.01$) (**Figure 4A**), indicating that GA treatment can prevent the proteasome activity from increasing in pressure overload hypertrophic hearts. Proteasomal CT-like activity resides in the β5 subunit of the 20S proteasome [26]. Hence, we examined myocardial protein levels of the steady state β5 subunit, but no remarkable changes were discerned (*data not shown*), sug-

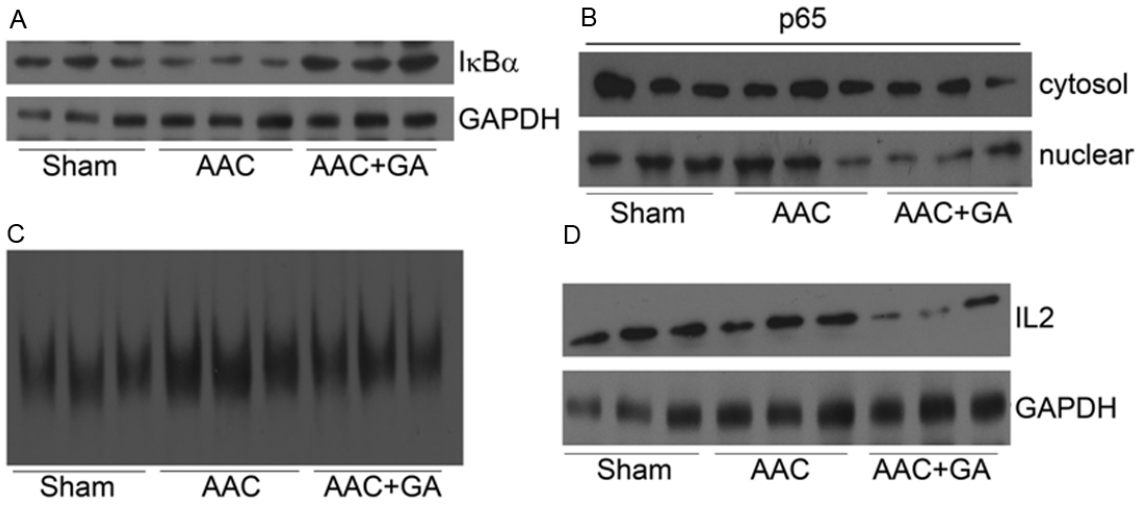

Figure 5. The effect of gambogic acid (GA) on myocardial NF-κB signaling in rats subject to abdominal aortic constriction (AAC). A: Western blot analysis for IκB in total myocardial protein extracts. B: Western blot analyses for the p65 subunit of NF-κB in the cytoplasmic fraction and the nuclear fraction of myocardial protein extracts. AAC or ISO increased nuclear translocation of NF-κB, which was attenuated by GA treatment. C: NF-κB DNA-binding activity examined by EMSA. D: Western blot analysis for myocardial levels of IL2 proteins. GAPDH was probe for loading controls.

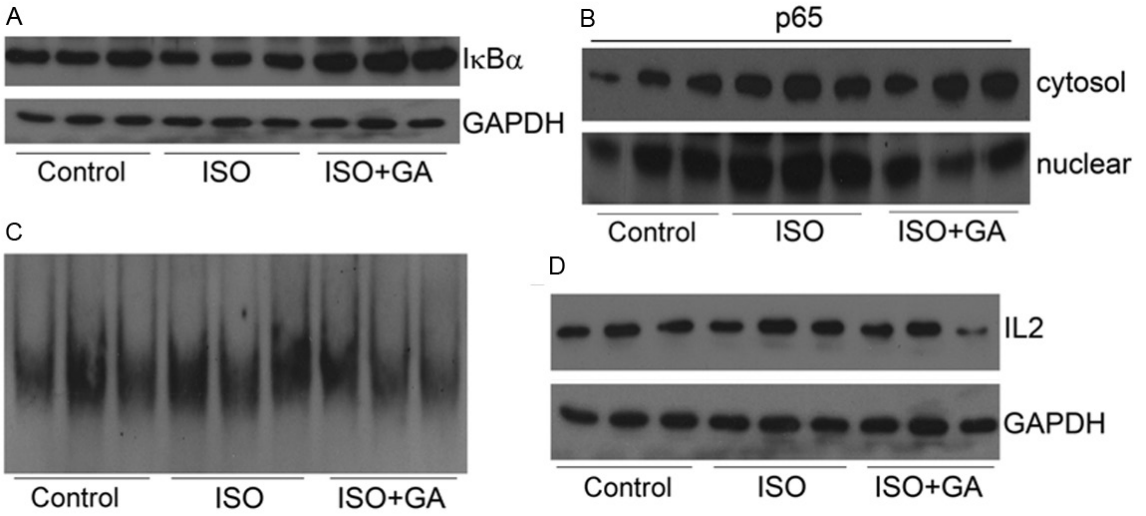

Figure 6. The effect of gambogic acid (GA) on myocardial NF-κB signaling in rats treated with isoproterenol (ISO). A: Western blot analysis for IκB in total myocardial protein extracts. B: Western blot analyses for the p65 subunit of NF-κB in the cytoplasmic fraction and the nuclear fraction of myocardial protein extracts. AAC or ISO increased nuclear translocation of NF-κB, which was attenuated by GA treatment. C: NF-κB DNA-binding activity examined by EMSA. D: Western blot analysis for myocardial levels of IL2 proteins. GAPDH was probe for loading controls.

gesting the increased CT-like activity is likely caused by factors other than increased 20S proteasome abundance. Similar findings were also obtained in the ISO rat model. ISO induced significant increases in myocardial proteasomal CT-like activity but the ISO-induced changes were all abolished by GA treatment. Interestingly, ISO also increased β5 subunit protein levels but the increase was abolished in the ISO+GA group (*data not shown*). These findings indicate that GA can suppress the increase of myocardial proteasome activity in the heart undergoing cardiac hypertrophy induced by either AAC-induced pressure overload or ISO infusion.

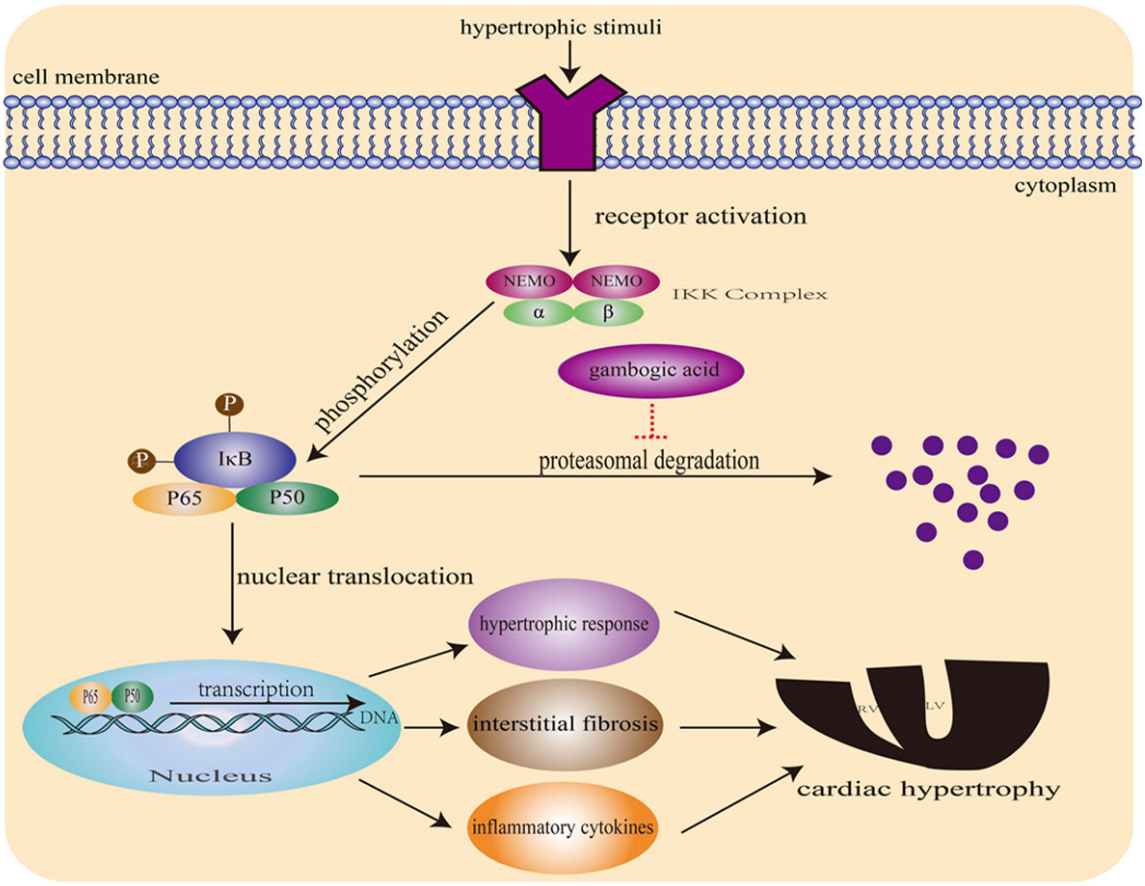

Figure 7. An illustration of a proposed mechanism by which gambogic acid suppresses cardiac hypertrophy and remodeling induced by pressure overload or isoproterenol infusion. Gambogic acid could increase the IκBα levels by the inhibition of proteasome activity, which blocks NF-κB translocation from the cytoplasm to the nucleus, thereby attenuating cardiac hypertrophy by decreasing hypertrophic gene expression, interstitial fibrosis and the levels of inflammatory cytokines (e.g. IL2).

Effects of GA on the NF-κB pathway in the heart during cardiac hypertrophy

Previous studies showed that the activation of NF-κB is essential for the development of cardiac hypertrophy induced by pressure overload *in vivo* [20]. Proteasome-mediated degradation of IκB allows the cytoplasmic NF-κB to translocate into the nucleus where NF-κB activates its target genes such as interleukin 2 (IL2); hence, inhibition of the proteasome may block the NF-κB signaling pathway [27, 28]. Since our data show that GA suppresses myocardial proteasome activities in both the pressure overload and the ISO induced cardiac hypertrophy models, we then examined changes in NF-κB signaling. Myocardial IκBα protein levels were substantially decreased in the AAC group but were marked increased in the AAC+GA group, compared with the sham control group (**Figure 5A**).

To further investigate the effects of GA on the nuclear translocation of NF-κB in hypertrophic cardiac tissue, which is an important step in the activation of NF-κB pathway, NF-κB p65 subunit levels in the nucleus and NF-κB DNA-binding activity were explored by western blotting analysis and EMSA, respectively. Nuclear NF-κB p65 subunit levels were increased in the AAC and the ISO groups, which were significantly inhibited by GA treatment (**Figure 5B**). EMSA reveals that NF-κB DNA binding was remarkably increased in the AAC group but not in the AAC+GA group (**Figure 5C**). Consistent with suppression of NF-κB pathway by GA, IL2 protein levels were significantly lower in the AAC+GA group compared with those in the sham or the

AAC groups (**Figure 5D**). Essentially the same results were also obtained in the ISO cohort (**Figure 6**). Myocardial IκB proteins were decreased in the ISO group but were considerably increased in the ISO+GA group, compared with the vehicle control group (**Figure 6A**). Compared with the vehicle control group, nuclear NF-κB proteins were markedly increased in the ISO group but decreased in the ISO+GA group (**Figure 6B**). Myocardial IL2 protein levels were lower in the ISO+GA group than other groups (**Figure 6D**). Taken together, these findings provide strong evidence that GA treatment inhibits the NF-κB signaling in rat hearts undergoing cardiac hypertrophy induced by AAC or ISO infusion, which may contribute to the anti-hypertrophy effects of GA.

Discussion

Multiple studies have shown cardiac hypertrophy as an independent risk factor for cardiovascular morbidity and mortality in humans [29]. Therefore, it is of high clinical significance to curtail cardiac hypertrophy. In the current study, we have used two cardiac hypertrophy models induced via AAC or isoproterenol infusion to demonstrate that GA can effectively reduce cardiac hypertrophic responses possibly through inhibition of the proteasome and blockade of the NF-κB pathway. The proposed mechanism by which GA suppresses cardiac hypertrophy is illustrated in **Figure 7**.

Cardiac hypertrophy involves the increases in both protein synthesis and degradation, although the rate of protein synthesis must be greater than protein degradation to achieve a net increase in protein and cardiac mass, characteristic of cardiac hypertrophy [30, 31]. The protein degradation in the heart, as in any other tissues or organs, is primarily performed by the proteasome. Alterations in myocardial proteasome function have been associated with a variety of heart disease in humans and animal models [32, 33]. Here we show that proteasomal peptidase activities, at least the CT-like activity, were remarkably increased during cardiac hypertrophy induced by either AAC-triggered pressure overload or ISO infusion (**Figure 4**). In agreement with our findings, previous reports showed that the proteasome activity is increased in the process of cardiac hypertrophy induced by pressure overload or ISO infusion [5, 6, 34]. The increased CT-like activity in AAC induced hypertrophic hearts is caused by factors other than increased proteasome abundance because the β5 subunit is not upregulated (*data not shown*). ATP is known to be essential for both ubiquitination and proteasomal degradation, but it has also been demonstrated that physiological levels of ATP negatively regulate proteasome activity; hence, the proteasome activity increases when the concentration of ATP in the cell declines to some extent [35, 36]. Therefore, the reduction of ATP generation in cardiomyocytes, as often seen in the hypertrophic heart, might have contributed to the increased proteasome activity. Many posttranslational modifications of the proteasome can also enhance its activity [37, 38]; hence, the increased proteasome peptidase activity could also be due to direct posttranslational modifications occurred to the proteasome.

Importantly, the increases in myocardial proteasome activities in both the AAC and ISO induced cardiac hypertrophy models were completely abolished by the GA treatment (**Figure 4**). This is accompanied by attenuation of hypertrophic responses and reduction of interstitial fibrosis by GA treatment (**Figures 1**-**3**). Given that proteasome inhibition using *bona fide* proteasome inhibitors was previously shown to suppress pressure overload cardiac hypertrophy and promote the regression of cardiac hypertrophy [7, 9, 10, 39, 40], the proteasome inhibition action of GA might have contributed to GA's anti-hypertrophy effects in these models. On the other hand, one alternative interpretation is that the GA-induced prevention of myocardial proteasome CT-like activity increase in rats subjected to AAC or ISO infusion might result from a proteasome-independent mechanism, such as inhibition of the NF-κB pathway via direct interaction and suppression of an IκB kinase [14], that suppresses cardiac hypertrophy. It is not known if GA could inhibit proteasome activities in the heart at baseline condition, admittedly a limitation of the present study.

Numerous stimuli and signaling pathways can be involved in the process of cardiac hypertrophy, and thus the underlying mechanisms in cardiac hypertrophy are very complex. The NF-κB pathway has been shown to mediate cardiac hypertrophy and maladaptive remodeling [41]. The UPS can regulate the activation of

NF-κB pathway at multiple steps [42, 43]; proteasome inhibition may thus suppress cardiac hypertrophy by inhibiting NF-κB activation. To better understand the molecular targets of GA on cardiac hypertrophy, we examined the NF-κB pathway in this study. We found that myocardial protein levels of IκBα, a key negative regulator of NF-κB activation, were decreased in rats subject to AAC or ISO infusion but the decrease was effectively reversed by GA treatment. The GA treatment even further raised the IκB protein levels in AAC or ISO rats beyond the baseline level (**Figures 5A**, **6A**), suggesting that GA can inhibit NF-κB activity not only during AAC or ISO induced hypertrophy, but at baseline as well. Indeed, among the three groups in both the AAC cohort and the ISO cohort, the GA treated groups showed the lowest nuclear NF-κB p65 subunit protein levels (**Figures 5B**, **6B**), the lowest NF-κB DNA-binding activity as revealed by EMSA (**Figures 5C**, **6C**), and the lowest protein levels of myocardial IL2 (**Figures 5D**, **6D**), an inflammatory cytokine that is stimulated by NF-κB activation [44]. These findings strongly support that the NF-κB pathway is activated by AAC or ISO and this activation is completely blocked by GA treatment.

In cardiac hypertrophy, several fetal genes, including α-skeletal actin and BNP, are reactivated. One proposed mechanism for increased BNP gene expression in cardiac hypertrophy *in vivo* is the activation of the NF-κB pathway [45]. Our data also showed that the relative expression levels of α-SK-actin and BNP mRNA in the hypertrophic cardiac tissue were inhibited by GA treatment (**Figure 3**). In addition, activation of the NF-κB pathway has been demonstrated to mediate cardiac fibrosis, an integrated part of maladaptive cardiac remodeling [8]. Our experiments showed that increased interstitial fibrosis in pressure overload (**Figure 2C**) or ISO infusion (**Figure 2D**) groups was attenuated by GA. Hence, the fetal gene expression data as well as the histopathology data are also consistent with the notion that suppression of the NF-κB pathway is likely a major mechanism underlying the suppression of pathological cardiac hypertrophy and maladaptive remodeling by GA.

In summary, our present study demonstrates for the first time that GA can block AAC or ISO from inducing cardiac hypertrophy and interstitial fibrosis, likely through inhibition of the proteasome and the NF-κB pathway (**Figure 7**). Although proteasome inhibition can lead to suppression of NF-κB activation, the experiments performed in the present study do not allow deciphering a cause-effect relationship between the two. It should be pointed out that numerous stimuli and signaling pathways contribute to the development of cardiac hypertrophy and pathology in response to AAC or ISO infusion and that GA may impact the heart, and perhaps peripheral vasculature, via other known and unknown actions. For example, administration of proteasome inhibitor bortezomib was recently shown to block angiotensin-II infusion from increasing blood pressure and attenuate angiotensin-II-induced vascular remodeling in rats [46]. Hence, we cannot rule out the possibility that changes in proteasome activities and the NF-κB pathway in the GA-treated groups could be the result of attenuation of the pathology by GA's actions beyond the heart. Regardless of detailed mechanisms, the unequivocal protection of GA on cardiac pathological hypertrophy and remodeling favors further investigation into the potential use of GA to treat cardiac disease.

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Disclosure of conflict of interest

None declared.

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