CTRP3/AMPK pathway plays a key role in the anti-hypertrophic effects of cyanidin-3-O-glucoside by inhibiting the inflammatory response

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Abstract

Background. Cardiac hypertrophy can be a pathological process that impairs heart function. Anthocyanins are a well-characterized type of natural antioxidant, and recent studies have shown that this type of compound has potential cardioprotective effects against different disorders, such as cardiac hypertrophy.

Objectives. We assessed the anti-hypertrophy potential of cyanidin-3-O-glucoside (C3G) and the mechanism associated with any observed effects.

Materials and methods. Hypertrophy symptoms were induced using the transverse aortic constriction (TAC) operation in vivo and angiotensin II (Ang II) in vitro. The effect of C3G on the development of hypertrophic symptoms was then determined. Moreover, we examined the influence of CTRP3 inhibition on the anti-hypertrophy function of C3G.

Results. The TAC operation induced cardiac fibrosis and heart weight increase, which was associated with increased production of cytokines and suppressed activity of the CTRP3/AMPK pathway. The impairments of heart structure and function were attenuated by C3G. Angiotensin II induced size increases of neonatal rat cardiomyocytes (NRCMs) in vitro, and this effect was inhibited by C3G. Furthermore, the inhibition of CTRP3 counteracted the function of C3G by promoting NRCM hyperplasia and inflammation.

Conclusions. The results of the current study showed that the activation of CTRP3 contributed to the anti-hypertrophy effects of C3G.

Key words: inflammation, AMPK, cyanidin-3-O-glucoside, cardiac fibrosis, CTRP3

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Background

Cardiovascular diseases are the primary cause of mortality in affluent countries and have gradually replaced smoking as the leading cause of death in underdeveloped countries.[1] Cardiac hypertrophy, an adaptive reaction of the heart, is common to several cardiovascular diseases.[2] Long-term myocardial hypertrophy predisposes the heart to damage by ischemia, arrhythmias, heart failure, and sudden death. As a result, early management of ventricular hypertrophy has garnered increased interest in recent years.

In developed countries, the use of diets for the treatment of chronic health issues has recently gained popularity. Anthocyanins are a subclass of flavonoids found in foods, such as fruits and vegetables, and are one of the most well-known functional chemicals.[3] These substances have antioxidative and anti-inflammatory characteristics, with several positive effects on human health.[3] Aloud et al. demonstrated that cyanidin-3-O-glucoside (C3G) has protective effects in cardiac diseases as it reduced the development of unfavorable ventricular hypertrophy in a hypertensive rat model,[4] confirming the anti-hypertrophy potential of anthocyanins.

Additionally, emerging evidence has demonstrated that the chronic inflammatory response plays a significant role in the pathogenesis of myocardial hypertrophy.[5,6] This notion is supported by the finding that individuals with heart failure have higher plasma levels of oxidative and pro-inflammatory cytokines.[7,8] For example, the development of cardiac hypertrophy is linked to the activation of pro-inflammatory transcription factors, including nuclear factor kappa B (NF-κB).[9] Given that anthocyanins are well-characterized anti-inflammatory agents, their ability to prevent hypertrophy may also be attributed to their anti-inflammatory effects.

There has been recent interest in the ability of certain natural compounds to affect upstream signaling pathways that modulate the immune response, and hence potentially prevent cardiac hypertrophy.[10,11] The CTRP3 is a member of C1q/tumor necrosis factor (TNF)-related proteins and is widely expressed in different tissues, such as white adipose tissue, fibroblasts, chondrocytes, and monocytes.[12,13] It possesses multiple biological functions, such as regulating adipokine secretion,[14] promoting cell proliferation,[15] regulating hepatic lipid metabolism,[16] and inhibiting the inflammatory response.[17] The activation of CTRP3 can reduce post-infarct cardiac fibrosis by activating Smad3 and preventing myofibroblast differentiation,[18] and the activity of the factor can be modulated by different natural compounds such as astragaloside IV and glycine monoester.[19,20] However, it is unclear whether the anti-inflammatory function of CTRP3 also contributes to the therapeutic mechanism of anthocyanins in the treatment of cardiac fibrosis and hypertrophy.

Objectives

In this study, we performed a preliminary investigation regarding the anti-hypertrophy effects of C3G by focusing on the inflammation-related CTRP3 pathway. The hypertrophic symptoms were induced in vivo using the transverse aortic constriction (TAC) method and in vitro using angiotensin II (Ang II). Then, the symptoms were treated with C3G, and the effects were analyzed using a series of assays to reveal the role of CTRP3-mediated anti-inflammatory effects in the anti-hypertrophy function of C3G.

Materials and methods

Establishment of transverse aortic constriction model

Eight-week-old male Sprague Dawley rats (approximate weight: 200 g) were bought from Huafukang Bioscience Co. Inc. (Beijing, China) and housed at 22°C with free access to food and water under a 12:12 h light–dark cycle. The Ganzhou People's Hospital Ethics Committee approved the animal experiments (approval No. 2020AN077). The study was performed following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) recommendations for animal studies. All animal experiments were carried out following the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

For all rats in the TAC and treatment groups, surgery was carried out to induce cardiac hypertrophy. Briefly, a 27-gauge needle was positioned next to the aorta between the right and left carotid arteries. After placing the ligature, the needle was removed, resulting in a ligation of 60–75% of the vessel diameter. To assess the effects of C3G on the progression of cardiac hypertrophy, 30 rats were randomly divided into 5 groups. The sham group was subjected to TAC surgery without constriction of the aorta and then administered normal saline (1 mL/day) via gavage for 8 weeks. In the TAC group, rats were subjected to TAC surgery and then gavaged with normal saline (1 mL/day) for 8 weeks. In the TAC+L group, rats were subjected to TAC surgery and then gavaged with 50 mg/kg body weight of C3G for 8 weeks.[21] Finally, the TAC+H group rats were subjected to TAC surgery and then administered C3G (100 mg/kg body weight) via gavage for 8 weeks.[21]

Cardiac function measurement

Upon completion of the experiment, the cardiac function of rats in different groups was assessed based on left ventricular end-systolic pressure (LVESP) and left ventricular end-diastolic pressure (LVEDP) while the rats were awake using a noninvasive blood pressure system (XBP 1000; Kent Scientific, Torrington, USA). The factional shortening of rats was measured using a Philips SON05500

system (Philips Ultrasound, Bothell, USA). Thereafter, cardiac tissues were obtained after the rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg). The ratio of heart weight to body weight was measured. The cardiac tissues were transected, fixed with 10% neutral formalin, and preserved at –80°C for subsequent assays.

Masson staining

To evaluate collagen deposition, tissues were extracted from the ventricles and exposed to Masson's trichrome analysis. Briefly, the sections were dehydrated using different concentrations of alcohol and embedded in paraffin. Three distinct dyes were used to distinguish between cells and the extracellular matrix. Tissues were incubated with hematoxylin solution for 6 min, ponceau and acid fuchsin solution for 1 min and phosphomolybdic acid solution for 5 min. Then, the tissues were re-stained with aniline blue solution for 5 min. The fibrotic area was determined using images obtained with a microscope (BX53; Olympus Corp., Tokio, Japan) at ×200 magnification. With this staining, collagens stain blue and muscle fibers stain red.

Detection of cytokine production

The levels of cytokines, including interleukin (IL)-6 (H007; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), IL-1β (H002; Nanjing Jiancheng Bioengineering Institute) and TNF-α (H052; Nanjing Jiancheng Bioengineering Institute) were measured using the corresponding enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions. Tissues or cells were homogenized, and 20 μL of the sample was incubated with 80 μL of a particular detecting solution at 37°C for 2 h. Thereafter, the supernatant was removed, and the mixture was incubated with 100 μL of horseradish peroxidase (HRP)-labeled streptavidin at 37°C for another 45 min. The cytokine levels were measured using the optical density (OD) values at 450 nm and 570 nm.

Western blotting

Myocardial tissues or neonatal rat cardiomyocytes (NRCMs) were homogenized using a lysis buffer to collect the total proteins. The protein concentration was determined using a bicinchoninic acid (BCA) protein concentration detection kit (ST506; Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Equal quantities of protein (40 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were incubated with primary antibodies (Supplementary Table 1) at 4°C overnight. Subsequently, secondary goat-anti-rabbit IgG-HRP antibodies (1:5000) (A0208; Beyotime Technology) were added to the membranes. The membranes were incubated at 37°C for 45 min. Following the visualization of protein bands using the BeyoECL Plus reagent (Beyotime Technology), the integral OD of the bands was recorded using the Gel-Pro-Analyzer (Media Cybernetics, Silver Spring, USA). The relative expression levels of the proteins were calculated with reference to the control group.

Preparation of neonatal rat cardiac myocytes and grouping

Neonatal Sprague Dawley rats (1–3 days old) were euthanized using pentobarbital sodium. The primary culture of NRCMs was performed according to the previously published method.[22] The cells were seeded at a density of 5×10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 mM 5-bromodeoxyuridine. To determine the role of CTRP3 in the anti-hypertrophy effect of C3G, NRCMs were classified into 4 groups. These included the control group, with normal NRCMs, the Ang II group, in which NRCMs were incubated with Ang II (1 μmol/L) for 48 h to induce hypertrophy, the Ang II + C3G group, in which NRCMs were pre-treated with 20 μM C3G 30 min before Ang II administration, and the Ang II + C3G + siRNA group, in which NRCMs were transfected with CTRP3 siRNA (Guangzhou RiboBio Company, Guangzhou, China) for 48 h before C3G and Ang II treatments.

Cell viability and surface area measurement

Following the Ang II administration, the supernatant of cultures was discarded. The NRCMs were incubated with the DMEM medium containing 10 μL Cell Counting Kit-8 (CCK-8) (HY-K0301, MedChem Express (MCE), USA) at 37°C for 1 h. The OD 450 value was detected using a microplate reader (ELX-800; BioTek, Santa Clara, USA) and used to indicate the cell viability. To investigate the development of hypertrophy, the cell surface area was evaluated under a microscope (BX53; Olympus Corp.) at a magnification of ×400.

Statistical analyses

Data are expressed as mean ± standard deviation (M ±SD). One-way analysis of variance (ANOVA) and post-hoc Tukey's test were performed. The normality and homogeneity of variance of different datasets were assessed with the Shapiro test and Levene's test, respectively, and the detailed results are shown in Supplementary Tables 2–19. Based on these analyses, all data met the normality and homogeneity of variance prerequisite for ANOVA. The significance level was set at <0.05 (two-tailed p-value). GraphPad Prism v. 6.0 (GraphPad Software, Inc., San Diego, USA) was used to perform statistical analyses and present histograms.

Results

C3G reduced the heart weight and improved the cardiac structure in TAC rats

The TAC method was used to induce hypertrophic cardiac symptoms. The successful establishment of the model was verified based on the increased values of LVEDP and reduced values of the LVESP and fractional shortening (FS) (Supplementary Fig. 1, Supplementary Table 1), which represented the characteristic features of hypertrophic hearts. Moreover, compared to the control and healthy groups, the TAC surgery significantly increased the ratio of heart weight to body weight (Fig. 1A, Table 1), confirming the hyperplasia of cardiac tissues. Furthermore, the changes in cardiac function and weight were related to the progression of fibrosis in heart tissues, where collagens were stained blue and muscle fibers were stained red (Fig. 1B). The collagen level was significantly higher in the heart tissues of the TAC group than in the control and sham groups. For rats administered with different doses of C3G, the heart weight to body weight ratio, as well as collagen deposition, was reduced (Fig. 1, Table 1). However, C3G treatment did not affect the hemodynamic parameters, irrespective of the dose used (Supplementary Fig. 1, Supplementary Table 20).

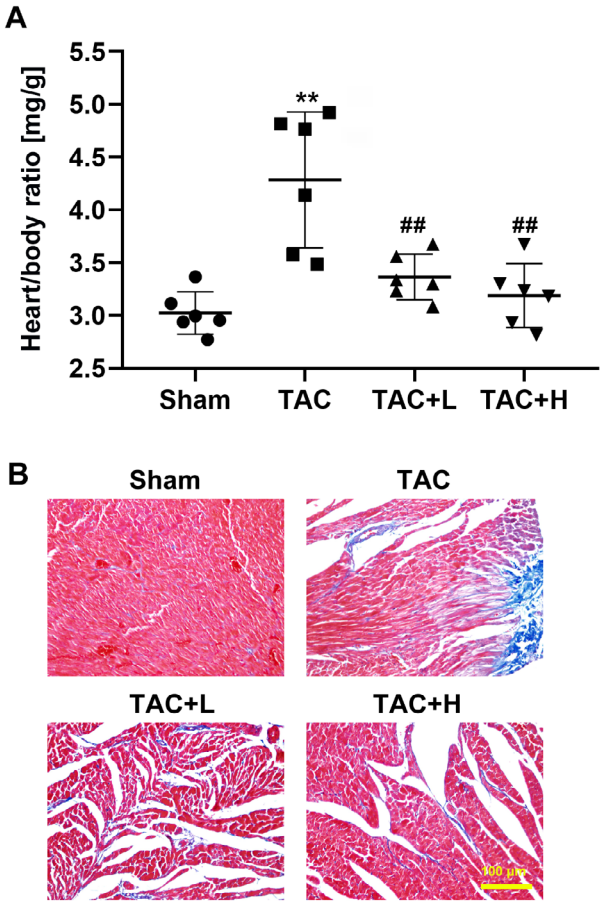

Fig. 1. Effects of cyanidin-3-O-glucoside (C3G) on heart weight and fibrosis in transverse aortic constriction (TAC) rats. Rats were subjected to TAC surgery to induce hypertrophy and then administered 2 doses of C3G (50 and 100 mg/kg body weight). A. Analysis results of heart/body ratio: C3G administrations decreased heart/body ratio; B. Masson staining of the collagen deposition: C3G administrations attenuated deposition of collagens; **p < 0.01 compared with the sham group; ##p < 0.01 compared with the TAC group. In the sham group, rats were subjected to TAC surgery without constriction of the aorta and then gavaged with normal saline (1 mL/day) for 8 weeks. In the TAC group, rats were subjected to TAC surgery and then gavaged with normal saline (1 mL/day) for 8 weeks. In the TAC+L group, rats were subjected to TAC surgery and then gavaged with 50 mg/kg body weight of C3G for 8 weeks. In the TAC+H group, rats were subjected to TAC surgery and then gavaged with 100 mg/kg body weight of C3G for 8 weeks

C3G inhibited the inflammatory response in TAC rats

The development of cardiac hypertrophy was significantly correlated with the inflammatory response, as evidenced by the increased production of IL-6, IL-1β and TNF-α (Fig. 2, Table 2). Moreover, the levels of cytokines were decreased after C3G treatment. Additionally, C3G had a dose-dependent effect on cytokine levels; treatment with 100 mg/kg body weight was associated with a higher anti-inflammatory effect than treatment with 50 mg/kg body weight (Fig. 2, Table 2).

C3G activated the CTRP3/AMPK pathway in TAC rats

The activity of the CTRP3/AMPK pathway was detected in rats with cardiac hypertrophy. The CTRP3 and p-AMPK/AMPK ratio levels in cardiac tissues were significantly lower after the TAC operation compared to the sham group (Fig. 3, Table 3). The changes in CTRP3/AMPK activity were restored by C3G administration.

Table 1. Effects of C3G on heart/body ratio

Group	Mean	SD	ANOVA with GLM		Tukey's test (p-value, q value)		
			p-value	F value	TAC	TAC+L	TAC+H
Sham	3.02	0.20	–	–	<0.0001, 8.02	0.4365, 2.17	0.8774, 1.06
TAC	4.28	0.64	–	–	–	0.0027, 5.85	0.0004, 6.97
TAC+L	3.36	0.22	–	–	–	–	0.8585, 1.12
TAC+H	3.19	0.30	<0.0001	12.84	–	–	–

C3G – cyanidin-3-O-glucoside; ANOVA – analysis of variance; TAC – transverse aortic constriction; L – low concentration; H – high concentration; SD – standard deviation; GLM – General Linear Model.

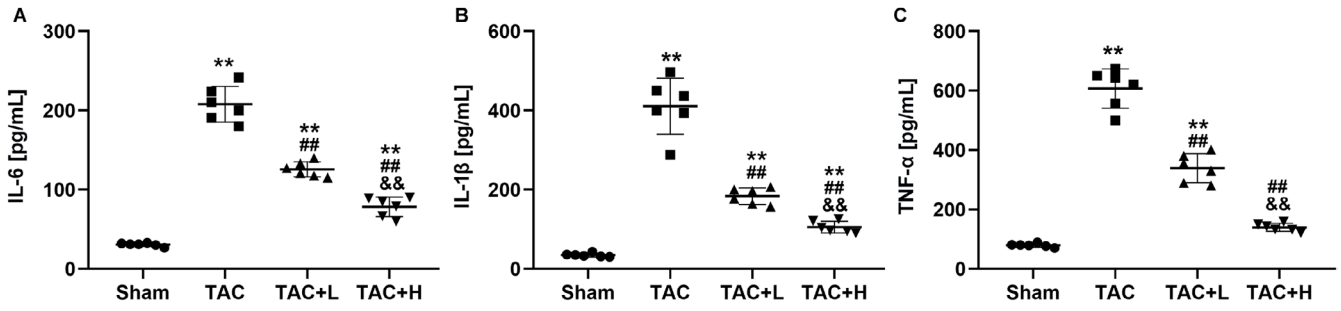

Fig. 2. Effects of cyanidin-3-O-glucoside (C3G) on cytokine production in myocardial tissues of transverse aortic constriction (TAC) rats. Rats were subjected to TAC surgery to induce hypertrophy and then administered 2 doses of C3G (50 and 100 mg/kg body weight). A. Analysis results of enzyme-linked immunosorbent assay (ELISA) detection of interleukin (IL)-6: C3G administrations decreased IL-6 level; B. Analysis results of ELISA detection of IL-1β: C3G administrations decreased IL-1β level; C. Analysis results of ELISA detection of tumor necrosis factor alpha (TNF-α): C3G administrations decreased TNF-α level; $^{**}p < 0.01$ compared with the sham group; $^{\#\#}p < 0.01$ compared with the TAC group; $^{\&\&}p < 0.01$ compared with the TAC+L group . In the sham group, rats were subjected to TAC surgery without constriction of the aorta and then gavaged with normal saline (1 mL/day) for 8 weeks. In the TAC group, rats were subjected to TAC surgery and then gavaged with normal saline (1 mL/day) for 8 weeks. In the TAC+L group, rats were subjected to TAC surgery and then gavaged with 50 mg/kg body weight of C3G for 8 weeks. In the TAC+H group, rats were subjected to TAC surgery and then gavaged with 100 mg/kg body weight of C3G for 8 weeks

Table 2. Effects of C3G on the production of cytokines in myocardial tissues

Parameter	Group	Mean [pg/mL]	SD	ANOVA with GLM		Tukey's test (p-value, q value)		
				p-value	F value	TAC	TAC+L	TAC+H
IL-6	sham	30.6	2.2	–	–	<0.0001, 31.54	<0.0001, 16.90	<0.0001, 8.49
	TAC	207.8	22.5	–	–	–	<0.0001, 14.64	<0.0001, 23.05
	TAC+L	125.6	9.5	–	–	–	–	<0.0001, 8.411
	TAC+H	78.3	12.4	<0.0001	180.7	–	–	–
IL-1β	sham	34.6	4.7	–	–	<0.0001, 24.44	<0.0001, 9.678	0.0089, 5.409
	TAC	410.8	70.8	–	–	–	<0.0001, 14.77	<0.0001, 19.84
	TAC+L	183.6	20.6	–	–	–	–	0.0092, 5.07
	TAC+H	105.5	14.7	<0.0001	112.5	–	–	–
TNF-α	sham	79.4	6.2	–	–	<0.0001, 30.99	<0.0001, 15.26	0.0895, 3.54
	TAC	607.3	66.1	–	–	–	<0.0001, 15.73	<0.0001, 27.45
	TAC+L	339.3	48.7	–	–	–	–	<0.0001, 11.71
	TAC+H	139.8	13.7	<0.0001	195.3	–	–	–

C3G – cyanidin-3-O-glucoside; IL – interleukin; TNF-α – tumor necrosis factor alpha; TAC – transverse aortic constriction; L – low concentration; H – high concentration; SD – standard deviation; GLM – General Linear Model.

Table 3. Effects of C3G on the expressions of proteins in CTRP3/AMPK pathway in myocardial tissues

Parameter	Group	Mean (IOD)	SD	ANOVA with GLM		Tukey's test (p-value, q value)		
				p-value	F value	TAC	TAC+L	TAC+H
CTRP3	sham	3148	38.5	–	–	<0.0001, 17.11	0.0003, 10.51	0.0082, 6.42
	TAC	1342	190.2	–	–	–	0.0082, 6.59	0.0070, 10.39
	TAC+L	2038	227.8	–	–	–	–	0.0773, 4.09
	TAC+H	2470	210.0	<0.0001	51.58	–	–	–
AMPK	sham	2819	115.8	–	–	0.0013, 8.668	0.0032, 7.49	0.0364, 4.85
	TAC	1729	106.6	–	–	–	0.8371, 1.18	0.1015, 3.82
	TAC+L	1676	78.1	–	–	–	–	0.3128, 2.64
	TAC+H	2134	87.5	0.0013	14.81	–	–	–
p-AMPK	sham	4018	161.0	–	–	<0.0001, 50.61	<0.0001, 48.97	<0.0001, 33.72
	TAC	1618	20.1	–	–	–	0.6656, 1.64	<0.0001, 16.89
	TAC+L	1696	22.3	–	–	–	–	<0.0001, 15.25
	TAC+H	2419	13.8	<0.0001	551.5	–	–	–

C3G – cyanidin-3-O-glucoside; IOD – integrated optical density; TAC – transverse aortic constriction; L – low concentration; H – high concentration; SD – standard deviation; GLM – General Linear Model.

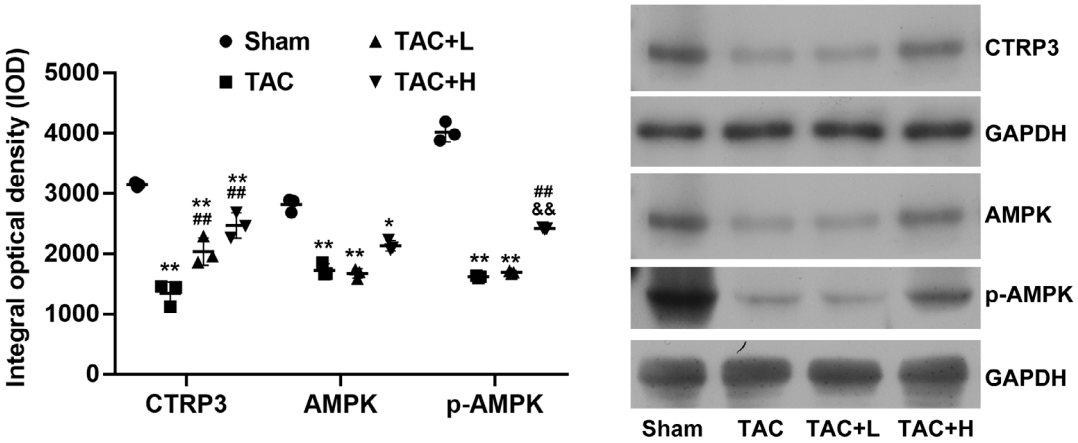

Fig. 3. Effects of cyanidin-3-O-glucoside (C3G) on the CTRP3/AMPK pathway in myocardial tissues in transverse aortic constriction (TAC) rats. Rats were subjected to TAC surgery to induce hypertrophy and then administered 2 doses of C3G (50 and 100 mg/kg body weight). Analysis results and images of western blotting detection of CTRP3, p-AMPK and AMPK levels: C3G administrations increased CTRP3 and p-AMPK levels; *p < 0.01 compared with the sham group; **p < 0.01 compared with the sham group; ##p < 0.01 compared with the TAC group; &&p < 0.01 compared with the TAC+L group. In the sham group, rats were subjected to TAC surgery without constriction of the aorta and then gavaged with normal saline (1 mL/day) for 8 weeks. In the TAC group, rats were subjected to TAC surgery and then gavaged with normal saline (1 mL/day) for 8 weeks. In the TAC+L group, rats were subjected to TAC surgery and then gavaged with 50 mg/kg body weight of C3G for 8 weeks. In the TAC+H group, rats were subjected to TAC surgery and then gavaged with 100 mg/kg body weight of C3G for 8 weeks

Moreover, the levels of CTRP3 and p-AMPK/AMPK ratio were higher in the treatment groups compared to the TAC group (Fig. 3, Table 3) Finally, the effect on the CTRP3/AMPK pathway was exerted in a dose-dependent manner.

C3G exerted anti-hypertrophy effects in NRCMs that were mediated via CTRP3 activation

To explore the mechanistic role of the CRTP3/AMPK axis in the anti-hypertrophy effects of C3G, the expression of CTRP3 in NRCMs was attenuated using siRNA (Fig. 4A, Table 4). Then, Ang II and C3G were administered in CTRP3-knockdown NRCMs. When compared to NRCMs transfected with NC siRNA, CTRP3 knockdown reduced the p-AMPK/AMPK ratio (Fig. 4A, Table 4) and the anti-hypertrophy effects of C3G were attenuated by alterations in CTPR3/AMPK pathway activity. Angiotensin II increased the proliferation potential and cell surface area (Fig. 4B,C; Table 5,6), as well as cytokine production in NRCMs, representing enhanced fibrosis (Fig. 4D–F, Table 7). In NRCMs co-treated with both Ang II and C3G, the proliferation potential and cytokine levels of NRCMs

Table 4. Effects of C3G on the expressions of proteins in CTRP3/AMPK pathway in NRCMs

Parameter	Group	Mean (IOD)	SD	ANOVA with GLM		Tukey's test (p-value, q value)		
				p-value	F value	Ang II	Ang II+C3G	Ang II+C3G+siRNA
CTRP3	control	4148	38.5	–	–	<0.0001, 81.61	<0.0001, 40.00	<0.0001, 57.35
	Ang II	1476	41.9	–	–	–	<0.0001, 41.62	<0.0001, 24.26
	Ang II+C3G	2838	37.5	–	–	–	–	<0.0001, 17.35
	Ang II+C3G+siRNA	2270	90.6	<0.0001	1181	–	–	–
AMPK	control	3880	100.0	–	–	<0.0001, 20.92	<0.0001, 13.06	<0.0001, 26.67
	Ang II	2677	121.9	–	–	–	0.0024, 7.86	0.0152, 5.95
	Ang II+C3G	3129	40.4	–	–	–	–	<0.0001, 13.61
	Ang II+C3G+siRNA	2346	115.0	<0.0001	133.3	–	–	–
p-AMPK	control	3106	85.4	–	–	0.0020, 8.09	0.0481, 4.57	0.9940, 0.36
	Ang II	2484	87.3	–	–	–	<0.0001, 12.66	0.0015, 8.45
	Ang II+C3G	3457	219.1	–	–	–	–	0.0686, 4.21
	Ang II+C3G+siRNA	3133	86.7	0.0001	28.0	–	–	–

ANOVA – analysis of variance; NRCMs – neonatal rat cardiac myocytes; Ang II – angiotensin II; C3G – cyanidin-3-O-glucoside; IOD – integrated optical density; GLM – General Linear Model.

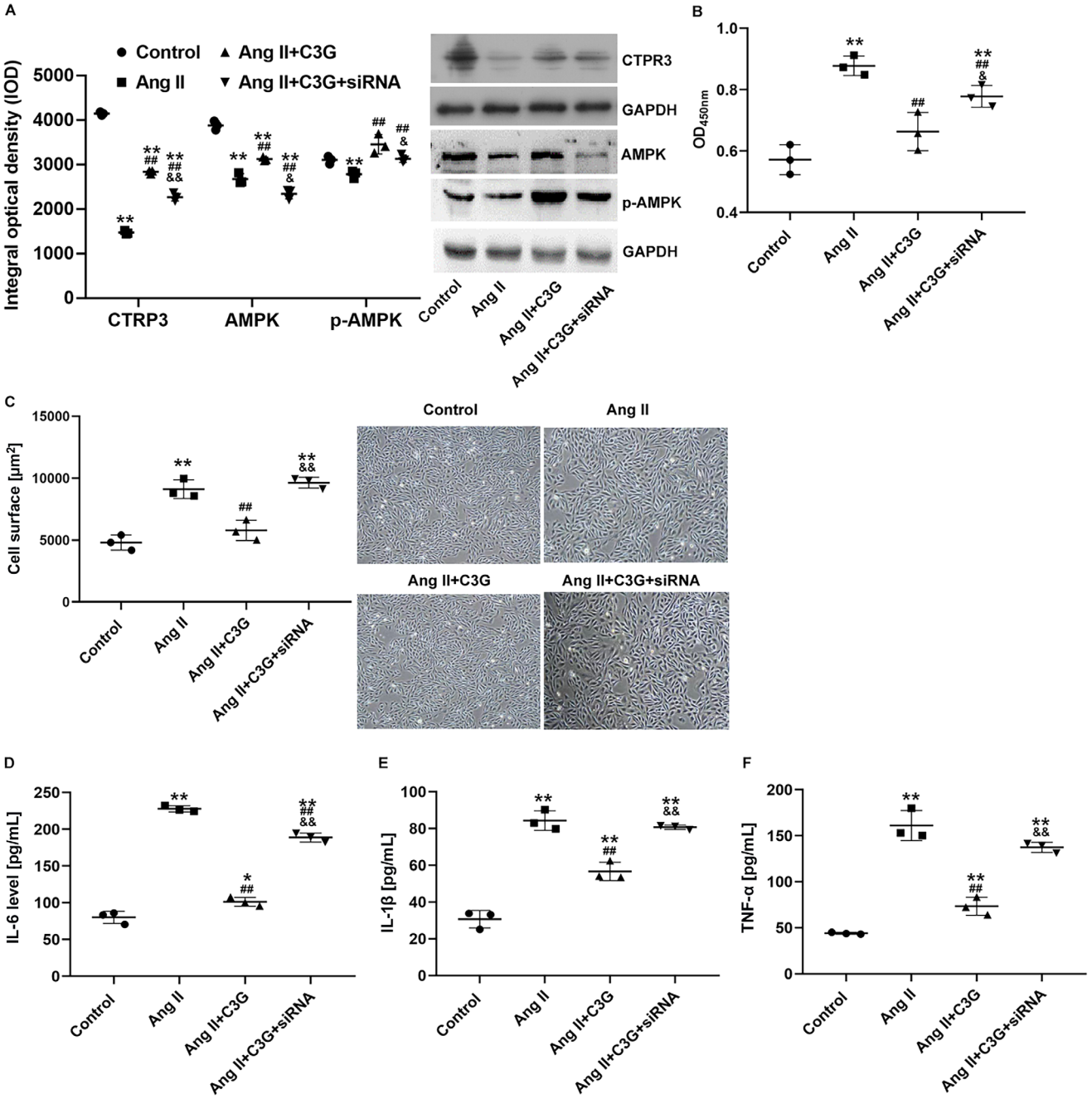

Fig. 4. Effects of CTRP3 inhibition on the anti-hypertrophy function of cyanidin-3-O-glucoside (C3G) in neonatal rat cardiac myocytes (NRCMs). NRCMs with CRTP3 inhibition were subjected to angiotensin II (Ang II) and C3G treatments. A. Analysis results and images of western blotting detection of CTRP3, p-AMPK and AMPK levels: inhibition of CTRP3 restored the level of p-AMPK even under C3G treatment; B. Analysis results of Cell Counting Kit-8 (CCK-8) detection of cell viability: inhibition of CTRP3 restored the viability of NRCMs even under C3G treatment; C. Analysis results of cell surface: inhibition of CTRP3 restored the surface area of NRCMs even under C3G treatment; D. Analysis results of enzyme-linked immunosorbent assay (ELISA) detection of interleukin (IL)-6: inhibition of CTRP3 restored IL-6 in NRCMs even under C3G treatment; E. Analysis results of ELISA detection of IL-1β: inhibition of CTRP3 restored IL-1β in NRCMs even under C3G treatment; F. Analysis results of ELISA detection of tumor necrosis factor alpha (TNF-α): inhibition of CTRP3 restored TNF-α in NRCMs even under C3G treatment; *$p < 0.05$ compared with the control group; **$p < 0.01$ compared with the control group; ##$p < 0.01$ compared with the Ang II group; &$p < 0.05$ compared with the Ang II + C3G group; &&$p < 0.01$ compared with the Ang II + C3G group. Control group, normal NRCMs; Ang II group, in which NRCMs were incubated with Ang II (1 μmol/L) for 48 h to induce hypertrophy; Ang II + C3G group, in which NRCMs were pre-treated with 20 μM C3G 30 min before Ang II administration; Ang II + C3G + siRNA group, in which NRCMs were transfected with CTRP3 siRNA for 48 h before C3G and Ang II treatments

were suppressed, further confirming the anti-fibrotic effects of C3G. Finally, the cell viability and cytokine levels in NRCMs were increased by CTRP3 inhibition, suggesting that C3G exerted anti-hypertrophy effects through CTRP3 activation.

Discussion

In this study, we demonstrated that long-term ingestion of C3G can reduce cardiac hypertrophy by delaying the fibrotic process and reducing inflammatory responses both

Table 5. Effects of C3G on the cell viability in NRCMs

Group	Mean (OD450)	SD	ANOVA with GLM		Tukey's test (p-value, q value)		
			p-value	F value	Ang II	Ang II+C3G	Ang II+C3G+siRNA
Control	0.572	0.05	–	–	0.0002, 11.51	0.1481, 3.43	0.0026, 7.76
Ang II	0.878	0.03	–	–	–	0.0020, 8.077	0.1087, 3.75
Ang II+C3G	0.663	0.06	–	–	–	–	0.0111, 5.327
Ang II+C3G+siRNA	0.778	0.03	0.0002	25.22	–	–	–

ANOVA – analysis of variance; NRCMs – neonatal rat cardiac myocytes; Ang II – angiotensin II; C3G – cyanidin-3-O-glucoside; SD – standard deviation; GLM – General Linear Model.

Table 6. Effects of C3G on the cell surface area in NRCMs

Group	Mean [μm^2]	SD	ANOVA with GLM		Tukey's test (p-value, q value)		
			p-value	F value	Ang II	Ang II+C3G	Ang II+C3G+siRNA
Control	4803	613	–	–	0.0002, 11.11	0.3433, 2.53	<0.0001, 12.47
Ang II	9105	752	–	–	–	0.0014, 8.58	0.7763, 1.35
Ang II+C3G	5783	816	–	–	–	–	0.0005, 9.93
Ang II+C3G+siRNA	9629	434	<0.0001	38.29	–	–	–

ANOVA – analysis of variance; NRCMs – neonatal rat cardiac myocytes; Ang II – angiotensin II; C3G – cyanidin-3-O-glucoside; SD – standard deviation; GLM – General Linear Model.

Table 7. Effects of C3G on the production of cytokines in NRCMs

Parameter	Group	Mean [pg/mL]	SD	ANOVA with GLM		Tukey's test (p-value, q value)		
				p-value	F value	Ang II	Ang II+C3G	Ang II+C3G+siRNA
IL-6	control	80.0	8.3	–	–	<0.0001, 40.38	0.0145, 5.80	<0.0001, 29.73
	Ang II	227.8	4.2	–	–	–	<0.0001, 34.58	0.0003, 10.65
	Ang II+C3G	101.3	6.1	–	–	–	–	<0.0001, 23.93
	Ang II+C3G+siRNA	188.8	6.1	<0.0001	369.2	–	–	–
IL-1β	control	30.7	4.8	–	–	<0.0001, 21.08	0.0004, 10.20	<0.0001, 19.63
	Ang II	84.4	5.3	–	–	–	0.0003, 10.88	0.7403, 1.45
	Ang II+C3G	56.7	5.0	–	–	–	–	0.0007, 9.43
	Ang II+C3G+siRNA	80.7	1.2	<0.0001	95.24	–	–	–
TNF-α	control	44.0	1.0	–	–	<0.0001, 20.46	0.0278, 5.125	<0.0001, 16.32
	Ang II	161.1	16.4	–	–	–	<0.0001, 15.33	0.0736, 4.142
	Ang II+C3G	73.3	9.7	–	–	–	–	0.0002, 11.19
	Ang II+C3G+siRNA	137.4	5.5	<0.0001	90.72	–	–	–

ANOVA – analysis of variance; IL – interleukin; TNF-α – tumor necrosis factor alpha; NRCMs – neonatal rat cardiac myocytes; Ang II – angiotensin II; C3G – cyanidin-3-O-glucoside; GLM – General Linear Model.

in vivo and in vitro. The anti-hypertrophy effects of C3G were attenuated by inhibiting the CTRP3/AMPK pathway because these effects are linked to CTRP3/AMPK pathway activation. The study results support the anti-hypertrophy effects of C3G, which are also supported by the findings of Aloud et al.[4] Moreover, the study provided an alternate explanation of the protective benefits of C3G against cardiac hypertrophy, which has long been overlooked. The establishment of the link between the anti-hypertrophy effects of C3G and the function of the CTRP3/AMPK axis forms the basis for the future development of adjuvant therapies and food items, based on the positive effects of C3G on improving cardiac hypertrophy. The mechanistic role of CTRP3/AMPK to drive the effects of C3G also explains the other biological activities of C3G and other anthocyanins. However, further studies are needed to explore this role.

Anthocyanins are a common class of water-soluble pigments that are characterized by their antioxidant,[23] anti-inflammatory,[24] neuroprotective,[25] and anti-diabetic properties.[26] With regard to the effects on the cardiovascular system, Liu et al. demonstrated that anthocyanin (C3G) protects against diabetes-related endothelial cell dysfunction by increasing adiponectin secretion.[27] Furthermore, Wang et al. demonstrated that C3G attenuates inflammation and apoptosis associated with endothelial

cell dysfunction by inhibiting the miR-204-5p/SIRT1 pathway.[21] Additionally, anthocyanins, such as C3G, have shown cardioprotective properties. Aloud et al. demonstrated that C3G can prevent hypertensive rats from developing maladaptive ventricular hypertrophy.[4] By administering C3G to Ang II-treated NRCMs and TAC rats, our study further confirmed its anti-hypertrophy effect. We demonstrated that C3G administration reduced inflammation and fibrosis, which are manifestations of cardiac hypertrophy. For the attenuation of other symptoms, such as cytokine production in myocardial tissues, the protective effects of C3G showed a dose-dependent effect. However, C3G had only a minor effect on hemodynamic parameters. Although our study used a prolonged treatment course and a high C3G treatment dose (up to 100 mg/kg), it is possible that the dose or duration of C3G treatment was insufficient to normalize the hemodynamic parameters. Additionally, because C3G is an active compound widely distributed in fruits, its effects may not be strong enough to induce significant functional changes in the heart, and this should be the focus of future investigations.

The results of the present and previous studies suggest that the anti-hypertrophy effects of C3G are closely related to its anti-inflammatory properties. In hypertrophic cardiac tissues, macrophage infiltration and inflammatory cytokine production are common.[28,29] Thus, anti-inflammatory medications can successfully reduce cardiac hypertrophy. For instance, astragaloside IV reduces the inflammatory response produced by the TLR4/NF-κB signaling pathway, which in turn reduces the myocardial hypertrophy caused by isoproterenol.[30] Yu et al. demonstrated that miR-143-3p inhibition ameliorates myocardial hypertrophy by inhibiting the inflammatory response.[31] As a well-characterized anti-inflammatory agent, anthocyanins, such as C3G, have positive effects on a variety of tissues, including the liver, gut and eyes. Our results support the previous findings that suggest that C3G can reduce inflammation in the heart.

Furthermore, the current study evaluated the alterations in the activity of the CTRP3/AMPK pathway to further explore whether the anti-inflammatory effects of C3G contribute to its anti-hypertrophy effect. The CTRP3 is a member of the CTRP family and is abundantly expressed in adipose tissue, heart and liver.[32] The CTRP3 can inhibit the inflammatory responses via several different mechanisms.[33,34] In the current study, we focused on changes in the CTRP3/AMPK pathway. The activation of the AMPK signaling transduction by CTRP3 can reduce cardiac dysfunction, inflammation, oxidative stress, and cell death in rats with diabetic cardiomyopathy.[18] Therefore, establishing the connection between C3G and CTRP3/AMPK may provide a preliminary explanation for the compound's anti-hypertrophy properties. Transverse aortic constriction and Ang II treatment reduced the CTRP3 level and p-AMPK/AMPK ratio, whereas C3G restored these levels both in vivo and in vitro, linking the reduction of hypertrophic symptoms to CTRP3/AMPK activation. Further evidence that the anti-hypertrophy effects of C3G are medicated by the activation of the CTRP3-mediated pathway was provided by the finding that inhibition of CTRP3 counteracted the function of C3G in NRCMs. As a result, the proliferation and inflammatory response were restored even under C3G treatment, which exacerbated the myocardial hypertrophy.

Limitations

The current study had certain limitations. First, the data only provided a preliminary explanation regarding the potential protective effects of anthocyanins against cardiac diseases. Second, the effects of C3G against cardiac hypertrophy were assessed using only a few assays, and a comprehensive analysis of the anti-hypertrophy effects was not performed. Thus, further studies are needed to investigate the potential effects of C3G and other anthocyanins against cardiac diseases.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.8385561. The package contains the following files:

Supplementary Fig. 1. Effects of C3G on hemodynamics parameters in TAC rats. Rats were subjected to TAC surgery to induce hypertrophy, and then handled with C3G of 2 doses. A. Analysis results of LVEDP; B. Analysis results of LVESP; C. Analysis results of FS. "**" $p < 0.01$ compared to sham group.

Supplementary Table 1. Antibody information.

Supplementary Table 2. Results of Shapiro test and Levene's test of heart/body ratio.

Supplementary Table 3. Results of Shapiro test and Levene's test of LVEDP.

Supplementary Table 4. Results of Shapiro test and Levene's test of LVESP.

Supplementary Table 5. Results of Shapiro test and Levene's test of FS.

Supplementary Table 6. Results of Shapiro test and Levene's test of IL-6 level in myocardial tissues.

Supplementary Table 7. Results of Shapiro test and Levene's test of IL-1β level in myocardial tissues.

Supplementary Table 8. Results of Shapiro test and Levene's test of TNF-α level in myocardial tissues.

Supplementary Table 9. Results of Shapiro test and Levene's test of CTRP3 level in myocardial tissues.

Supplementary Table 10. Results of Shapiro test and Levene's test of p-AMPK level in myocardial tissues.

Supplementary Table 11. Results of Shapiro test and Levene's test of AMPK level in myocardial tissues.

Supplementary Table 12. Results of Shapiro test and Levene's test of CTPR3 level in cells.

Supplementary Table 13. Results of Shapiro test and Levene's test of p-AMPK level in cells.

Supplementary Table 14. Results of Shapiro test and Levene's test of AMPK level in cells.

Supplementary Table 15. Results of Shapiro test and Levene's test of cell viability.

Supplementary Table 16. Results of Shapiro test and Levene's test of cell area.

Supplementary Table 17. Results of Shapiro test and Levene's test of IL-6 in cells.

Supplementary Table 18. Results of Shapiro test and Levene's test of IL-1β in cells.

Supplementary Table 19. Results of Shapiro test and Levene's test of TNF-α in cells.

Supplementary Table 20. Effects of C3G on hemodynamics parameters.

Conclusions

Our results support the hypothesis that C3G possesses anti-hypertrophy properties and could inhibit fibrosis and inflammation both in vitro and in vivo. This effect depends on the activation of the CTRP3/AMPK pathway. Our results add to the existing knowledge regarding the use of anthocyanins in functional diets or as an adjunctive treatment to reduce cardiac hypertrophy.

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