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RESEARCH ARTICLE

Acetyl salicylic acid attenuates cardiac hypertrophy through Wnt signaling

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Abstract **Ventricular hypertrophy is a powerful and independent predictor of cardiovascular morbid events. The vascular properties of low-dose acetyl salicylic acid (aspirin) provide cardiovascular benefits through the irreversible inhibition of platelet cyclooxygenase 1; however, the possible anti-hypertrophic properties and potential mechanism of aspirin have not been investigated in detail. In this study, healthy wild-type male mice were randomly divided into three groups and subjected to transverse aortic constriction (TAC) or sham operation. The TAC-operated mice were treated with the human equivalent of low-dose aspirin (10 $mg \cdot kg^{-1} \cdot d^{-1}$); the remaining mice received an equal amount of phosphate buffered saline with 0.65% ethanol, which was used as a vehicle. A cardiomyocyte hypertrophy model induced by angiotensin II (10 $nmol \cdot L^{-1}$) was treated with the human equivalent of low (10 or 100 $\mu mol \cdot L^{-1}$) and high (1000 $\mu mol \cdot L^{-1}$) aspirin concentrations in plasma. Changes in the cardiac structure and function were assessed through echocardiography and transmission electron microscopy. Gene expression was determined through RT-PCR and western blot analysis. Results indicated that aspirin treatment abrogated the increased thickness of the left ventricular anterior and posterior walls, the swelling of mitochondria, and the increased surface area in *in vivo* and *in vitro* hypertrophy models. Aspirin also normalized the upregulated hypertrophic biomarkers, β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), and b-type natriuretic peptide (BNP). Aspirin efficiently reversed the upregulation of β-catenin and P-Akt expression and the TAC- or ANG II-induced downregulation of GSK-3β. Therefore, low-dose aspirin possesses significant anti-hypertrophic properties at clinically relevant concentrations for anti-thrombotic therapy. The downregulation of β-catenin and Akt may be the underlying signaling mechanism of the effects of aspirin.**

Keywords aspirin; Akt; cardiac hypertrophy; GSK-3β; Wnt/β-catenin

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death among non-communicable diseases [1]. The hallmarks of CVD include cardiac hypertrophy and increased incidence of cardiovascular events, such as ischemic episode, arrhythmias, and sudden death [2,3]. Low-dose acetylsalicylic acid (aspirin) is commonly prescribed for patients with CVD because aspirin can be used to effectively treat and prevent primary and secondary cardiac events, and this treatment has been subjected to meta-analysis [4] and reviews [5–7]). Aspirin inhibits prostaglandin H synthetase (cyclooxygenase, COX-1) in platelets to decrease the synthesis of thromboxane A_2, a potent platelet stimulant and vasoconstrictor; thus, aspirin prevents the conversion of platelet-mediated atherosclerotic plaque fissuring repair into full vascular occlusion [5–7]. Nonetheless, aspirin yields a higher absolute risk

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reduction in the heart than in other organs, such as brain [4–7]; this finding suggests that aspirin may possess a "nonvascular" mode of action. We hypothesized that aspirin may also be beneficial through the attenuation of ventricular hypertrophy (VH). VH is a prognostic marker of CVD; VH is also an independent and powerful predictor of cardiac events [2,3,8].

Cancer epidemiological studies have suggested that aspirin possesses anti-proliferative properties [9–11]. Interestingly, the Wnt/β-catenin signaling cascade has been identified as an aspirin target in cancer prevention [9]; this cascade is also implicated in the development of pathological cardiac hypertrophy [12]. A hallmark of Wnt/β-catenin signaling is the nuclear translocation of β-catenin; in this process, β-catenin functions as a co-transactivator of the T cell factor/lymphocyte enhancer factor (TCF/LEF) to mediate VH [12]. This co-transactivator is regulated via a glycogen synthetase kinase 3β (GSK-3β)-dependent mechanism mediated through Wnt and Akt activation [13].

In the present study, the human equivalent of low-dose aspirin was administered to mice, and cardiomyocyte hypertrophy models were established with transverse aortic constriction (TAC) and angiotensin (ANG) II, respectively. Aspirin normalized the cardiac function and significantly reduced the hypertrophic phenotypes induced by TAC and ANG II, as evidenced by the normalized hypertrophic biomarkers, atrial natriuretic peptide (ANP), b-type natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC). Furthermore, aspirin efficiently reversed the upregulation of β-catenin and P-Akt expression and the phosphorylation of (Ser^{9}) GSK-3β.

In summary, low-dose aspirin partially attenuates cardiac hypertrophy through the downregulation of Wnt signaling. These findings strongly suggest that the attenuation of hypertrophy may contribute to the long-recognized benefits of low-dose aspirin to prevent and reduce the risk of cardiovascular events.

Materials and methods

Materials

Wild-type male mice (C57B6) and neonatal Wistar rats were obtained from the Second Affiliated Hospital, Harbin Medical University. Aspirin, Dickkopf-1 (DKK), and ANG II were purchased from Sigma (Sigma-Aldrich); β-catenin, Lamin B1, and phospho-Akt (Ser^{473}) were obtained from Cell Signaling; GSK-3β (phospho Ser^{9}) was procured from Abcam; β-MHC and GAPDH were purchased from Santa Cruz Biotechnology. The primer pairs for ANP, BNP, β-MHC, β-catenin, and GAPDH were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China).

Oral aspirin (1 $mg \cdot ml^{-1}$) solution for *in vivo* administration

Aspirin crystals were weighed (30 mg) and dissolved in 150 µl of absolute ethanol. Afterward, 30 ml of PBS was added to the solution to prepare 1 $mg \cdot ml^{-1}$ aspirin solution.

In vivo aspirin treatment in mice

This study was approved by the Institutional Animal Care and Use Committee of Harbin Medical University, China and was in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

A total of 60 three-month-old mice, weighing between 20 and 30 g, were allowed to acclimatize for a week under standard animal room conditions (temperature, 25 °C; humidity, 55%–60%; 12 h light-dark cycle) with *ad libitum* access to food and water. The mice were randomly divided into three groups ($n = 20$) for the subsequent experiments.

The potential anti-hypertrophic effects of aspirin was evaluated with the 75 $mg \cdot d^{-1}$ dose (equivalent to 1 $mg \cdot kg^{-1} \cdot d^{-1}$ in human with an assumed average human weight of 70 kg) [14] because aspirin produces optimal "anti-platelet" effects to prevent cardiovascular events, such as sudden death, angina, myocardial infarction (MI), or stroke [6,15]. The equivalent animal daily dosage (10 $mg \cdot kg^{-1} \cdot d^{-1}$) was derived from the body surface area [16]. The animals were weighed, and an appropriate dose of freshly prepared aspirin solution (1 $mg \cdot ml^{-1}$) was measured and administered through oral gavage. The control animals included non-aspirin-treated TAC and sham-operated mice that received an equivalent volume of the vehicle (phosphate buffered saline [PBS] + ethanol [0.65%]). The animals were treated for four consecutive days before TAC was conducted, and treatment was continued thereafter for one month after surgery was completed.

In vivo hypertrophy induction

TAC is a murine hypertrophy model that intimately simulates human cardiac response to hypertension or aortic valve stenosis [17]. The procedure was performed under deep systemic anesthesia of phenobarbital (50 $mg \cdot kg^{-1}$, intraperitoneal) [18], and the depth of anesthesia was evaluated through a tail or paw pinch. The mice were then intubated with an endotracheal cannula connected to a volume cycled rodent ventilator with a tidal volume of 50 ml and a respiratory rate of 100 per min (UGO BASILE S. R. L., Italy). TAC was then performed in accordance with previously described methods [19]. In brief, the aortic arch was identified after thoracotomy was performed. A 7.0-silk suture was placed

around the transverse aorta between the innominate and left carotid arteries and then ligated around a 26-gauge blunt needle, which was subsequently removed; as a result, systolic pressure was increased by 1.5-fold to induce VH [19]. The sham-operated control mice underwent the same surgical procedures, but the aorta of these mice was not ligated. The chest was then closed, and the animals were allowed to recover. After surgery was completed, buprenorphine hydrochloride (0.1 $mg \cdot kg^{-1}$, subcutaneous) was administered to induce analgesia. The same dose of buprenorphine was subsequently injected one day after surgery when the animals displayed signs of distress.

Echocardiography and tissue harvesting

The left ventricular (LV) function was assessed through transthoracic echocardiography at the end of the four-week treatment in accordance with previously described methods [20]. In brief, the mice were weighed, anesthetized as previously mentioned, and visualized in the left lateral decubitus position by using a Vivo 2100 rodent echocardiographic imaging system (Visualsonics, Toronto, Canada) equipped with a 30 MHz microscan transducer. The M- and B-mode tracings were both recorded in parasternal long and short axis views from the midpapillary muscle. The interior diameter of LV, the thickness of anterior and posterior walls, and the mass of LV were assessed; fractional shortening (FS) and ejection fraction (EF) were also evaluated.

The animals were subsequently sacrificed. The hearts were quickly harvested and weighed in ice-cold PBS. A portion of each heart was snap-frozen in liquid nitrogen and stored at −80 °C for subsequent western blot or real-time PCR analysis. The other portion was fixed in 4% buffered paraformaldehyde or 2.5% glutaraldehyde in 0.1 $mol \cdot L^{-1}$ PBS (pH 7.4) and then subjected to immunohistochemical analysis or electron microscopy, respectively. The length of the left tibia was measured and subjected to hypertrophy analysis.

Transmission electron microscopy

Transmission electron microscopy was performed on randomly selected LV sections as previously described [19]. In brief, the selected samples (5 nm) were fixed with 2.5% glutaraldehyde in 0.1 $mol \cdot L^{-1}$ PBS (pH 7.4) for 2 h, post-fixed in PBS buffer containing 1% osmium tetraoxide (OsO_4) for 1 h, and embedded as a monolayer. The sections were stained, blocked in uranyl acetate, dehydrated in ethanol, and embedded in epoxy resin in accordance with standard procedures. The ultrathin sections were stained and examined under an electron microscope (JEM-1220; JEOL, Ltd., Japan).

Primary neonatal rat cardiomyocyte culture

One- to three-day-old neonatal Wistar rats were anesthetized with phenobarbital (25 $mg \cdot kg^{-1}$, subcutaneous). The drug is efficacious and appropriate for rats younger than postnatal day 3 [21]. The rats were sacrificed through decapitation, and the hearts were isolated after thoracotomy was performed; the cardiac tissue was digested and then cultured in accordance with previously described methods [19,20]. In brief, heart tissue was digested in 0.25% trypsin at 37 °C. The isolated cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% FBS (Gibco) and 1% penicillin (100 $U \cdot ml^{-1}$)-streptomycin (100 $\mu g \cdot ml^{-1}$); the suspended cells were then cultured in a humidified incubator (95% air-5% CO_2) for 90 min to help fibroblasts attach to the culture plate. The isolated NRVMs were supplemented with 0.1 $mmol \cdot L^{-1}$ 5-bromo-2′-deoxyuridine (BRDU) to inhibit non-cardiomyocyte growth; afterward, NVRMs were seeded at a density of 3×10^5 cells per well in six-well plates or 6×10^5 cells per bottle in 4 ml culture flasks. The cells were incubated for 48 h to allow NVRM adherence; the culture medium was replaced with fresh DMEM/FBS and the appropriate treatment.

In vitro treatment with low aspirin concentration

The *in vitro* study simulated human low aspirin plasma concentrations in an ANGII-induced NRVM hypertrophy model. The NRVMs were treated with the animal equivalent of low (10 and 100 $\mu mol \cdot L^{-1}$) and high (1000 $\mu mol \cdot L^{-1}$) aspirin concentrations [16] in accordance with the reported peak plasma aspirin levels (C_{max}) in human volunteers on a 100 $mg \cdot d^{-1}$ dose [22,23]. The NVRMs were randomly divided into four groups: control (treated with DMSO at a final concentration of $< 0.01\%$); ANG II (10 $nmol \cdot L^{-1}$); aspirin (varying concentrations) + ANG II; and DKK (200 $nmol \cdot L^{-1}$) + ANG II.

The cells were initially incubated with aspirin (at the derived final concentrations) for 12 h or with DKK for 1 h; the incubated cells were then treated with aspirin or DKK + ANG II for 24 h. The cells were supplied with fresh drugs and medium; afterward, the cells were incubated for another 24 h.

Immunohistochemistry

NVRMs were immunohistochemically examined after *in vitro* experimental procedures were conducted in accordance with a previously described protocol [19]. In brief, NVRMs were fixed in 4% paraformaldehyde and permeabilized with Triton X plus BSA for 1 h. The samples were blocked with goat serum for 1 h and incubated with the primary antibody (anti-α actinin, 1:200; Sigma) for 12 h at

4 °C. The cells were then incubated with the secondary antibody for 1 h (Alexa Fluor 594, 1:1000; Invitrogen). The nuclei were counterstained with 4′,6-diamino-2-phenylindole (DAPI, 1:50; Invitrogen). Immunofluorescence was visualized under a fluorescence microscope (Nikon 80i), and 100 cardiomyocytes in 20–30 fields were examined for each group. Hypertrophy was evaluated on the basis of the relative α-actinin positive surface area measured by using the Image-Pro Plus (Version 5.0.1) software.

Protein extraction and western blot analysis

After the experimental procedures, the total protein lysates and the cytoplasmic and nuclear protein fractions were extracted from tissue and NVRMs for immunoblot analysis. The total protein lysate was extracted with the RIPA buffer (Beyotime Institute of Biotechnology, China) as previously described [20]. The cytoplasmic and nuclear proteins were extracted with the cytoplasmic and nuclear extraction buffers (Beyotime Institute of Biotechnology, China), respectively, by following the manufacturer's instructions. Immunoblot procedures were essentially identical to previous reports [19,20]. Briefly, the protein concentration was determined with the BCA Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions. Protein samples were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, USA) by electroelution. The membranes were blocked in 5% non-fat milk in PBS at room temperature for 2 h, and incubated overnight at 4 °C with the following primary antibodies: β-MHC (rabbit, 1:1000 dilution), β-catenin (rabbit, 1:1000), P-Akt (rabbit, 1:200), GSK-3β (rabbit, 1:1000), GAPDH (mouse, 1:500), or lamin B1 (rabbit, 1:500). Afterward, the membranes were washed thrice in PBS with 0.1% Tween 20 (PBST); each time interval was a minimum of 5 min. The membranes were further incubated with the fluorescence-conjugated goat anti-rabbit or mouse IgGs (1:10 000 dilution; Invitrogen) for 1 h at room temperature. Immunoblot bands were quantified and analyzed with Odyssey's Infrared Imaging System and v3.0 software (LI-COR).

Real-time PCR analysis

Total RNA was extracted from heart tissue and NVRMs with TRIzol reagent (Life Technologies) as previously described [20]. From each sample, 1.0 μg of total RNA was used to generate cDNA with High-Capacity cDNA reverse transcription kits (Applied Biosystems) in accordance with the manufacturer's instructions. Real-time PCR was performed with the SYBR Green PCR Master Mix and the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The amplification cycles included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The primers (Shanghai Sangon Biotech Co., Ltd. China) for β-MHC mRNA were 5′-CCAGAAGCCTC-GAAATGTC-3′ (forward, F) and 5′-CTTTCTTTGC-CTTGCCTTTGC-3′ (reverse, R); for ANP mRNA were 5′-CTCCGATAGATCTGCCCTCTTGAA-3′ (F) and 5′-GGTACCGGAAGCTGTTGCAGCCTA-3′ (R); for BNP mRNA were 5′-TGATTCTGCTCCTGCTTTTC-3′ (F) and 5′-GTGGATTGTTCTGGAGACTG-3′ (R); for β-catenin mRNA were 5′-ATCATTCTGGCCAGTGGTGG-3′ (F) and 5′-GACAGCACCTTCAGCACTCT-3′ (R); for GAPDH mRNA were 5′-GGGGCTCTCTGCTCCTCC-CTG-3′ (F) and 5′-CGGCCAAATCCGTTCACACCG-3′ (R). The transcript quantities were compared by the relative quantitative method, where the amount of detected mRNA was normalized to the amount of the internal control, GAPDH. The relative value to the control sample was determined by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.01, and data were presented as mean ± SEM. The significance of differences between mean values was determined by Student's *t*-test or ANOVA followed by post-hoc Bonferroni correction test for multiple comparisons. Three independent experiments were performed for each test, and $P < 0.05$ was considered significant.

Results

Aspirin attenuates cardiac hypertrophy *in vivo*

After the treatment was administered, the ventricular systolic and diastolic structures and functions were assessed through echocardiography. As illustrated in Fig. 1A and 1B as well as Table 1, aspirin treatment preserved normal ventricular function, whereas the TAC group developed significantly reduced chamber size, increased wall thickness, and increased % FS and % EF, which are consistent with concentric hypertrophy [24]. Deleterious ultrastructural changes that preceded gross morphological alterations typical of VH were observed through transmission electron microscopy. As shown in Fig. 1C, the TAC group exhibited a marked cellular derangement evidenced by the irregular *Z* lines, damaged and swollen mitochondria, and the increased convolution of intercalated discs. These anomalies were normalized by aspirin treatment. Furthermore, aspirin significantly reduced the TAC-induced heart enlargement, as indicated by the diminished heart-to-body weight ratio (4.5 ± 0.09 $mg \cdot g^{-1}$, $n = 10$ for aspirin + TAC vs. 5.0 ± 0.09 $mg \cdot g^{-1}$, $n = 13$ for TAC alone) and heart weight-to-tibia length ratio (6.1 ± 0.15 $mg \cdot mm^{-1}$, $n = 10$ for aspirin vs. 7.5 ± 0.16

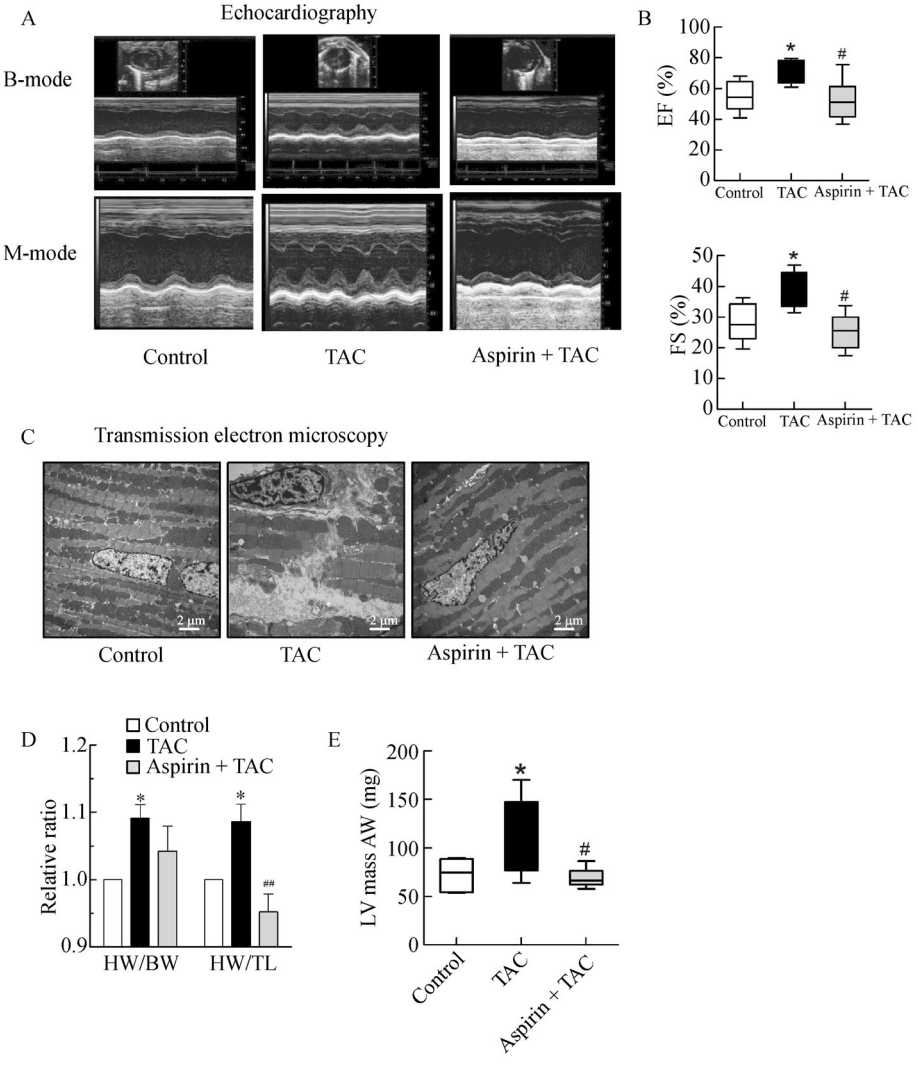

Fig. 1 Effect of aspirin on cardiac morphology and function. (A) Representative echocardiographic images of ventricular myocardium, including the B-mode (upper panel) and M-mode (lower panel) recordings. The number (n) of mice analyzed per group is given in Table 1. Aspirin (10 $mg \cdot kg^{-1} \cdot d^{-1}$) restored the TAC-induced decrease in chamber size and the increase in wall thickness. (B) Box and whisker plots summarizing the changes in the ejection fraction (% EF) and fractional shortening (% FS). Aspirin normalized cardiac function. The data were presented as mean ± SEM; $n = 6$; $^{\#}P < 0.05$ vs. TAC; * $P < 0.05$ vs. Control. (C) Representative electron micrographs for the ultrastructural analysis of the ventricular myocardium. No tissue swelling occurred in the control group. By contrast, the non-treated TAC group displayed marked degenerative changes as indicated by swollen mitochondria with amorphous matrix densities, distended myofibrils, and irregular Z lines. However, the aspirin-treated group had fewer and smaller alterations; $n = 3$; magnification, 15 000 × ; HV, 80 kV; scale bar calibration, 2 μm. (D) Bar graph summarizing the heart weight (HW) normalized to body weight (BW) or tibia length (TL). The data were presented as mean ± SEM; n is given in the text; $^{\#\#}$ $P < 0.0001$ vs. TAC; * $P < 0.05$ vs. Control. (E) Bar graph summarizing the measured left ventricular mass based on the M-mode echocardiography. The data were presented as mean ± SEM; $n = 6$; $^{\#}P < 0.05$ vs. TAC; * $P < 0.05$ vs. Control. Control, sham-operated mice treated with vehicle (PBS + 0.65% ethanol); TAC, TAC-operated mice treated with vehicle; Aspirin + TAC, TAC-operated mice treated with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ aspirin (human equivalent dose of 1 $mg \cdot kg^{-1} \cdot d^{-1}$).

$mg \cdot mm^{-1}$, $n = 13$, for TAC alone; $P < 0.001$; Fig. 1D). Similar results after aspirin treatment were observed with the M-mode echocardiographic analysis of the left ventricular mass (Fig. 1E).

At the molecular level, aspirin significantly down-regulated the mRNA levels of several biomarkers of cardiac hypertrophy, including β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), and b-type natriuretic peptide (BNP; Fig. 2A), as well as the protein expression of β-MHC (Fig. 2B).

Table 1 Effects of aspirin ($10\ mg \cdot kg^{-1} \cdot d^{-1}$) on cardiac function, as measured by echocardiography

		Control ($n = 6$)		TAC ($n = 13$)			Aspirin + TAC ($n = 7$)		
		mean	SEM	mean	SEM	*P	mean	SEM	# P
LVAW; d	mm	0.74	0.16	1.01	0.24	0.020	0.76	0.25	0.034
LVAW; s	mm	1.07	0.27	1.39	0.31	0.045	1.08	0.29	0.042
LVID; d	mm	3.74	0.47	3.23	0.46	0.035	3.69	0.43	0.040
LVID; s	mm	2.71	0.55	2.02	0.46	0.010	2.69	0.59	0.010
LVPW; d	mm	0.69	0.10	1.14	0.48	0.049	0.59	0.13	0.048
LVPW; s	mm	0.98	0.17	1.37	0.38	0.029	0.91	0.24	0.009
LV Vol; d	µl	60.95	16.72	43.12	13.79	0.023	58.61	15.26	0.030
LV Vol; s	µl	28.84	13.27	14.24	7.27	0.005	28.58	13.91	0.005

Note: Summary of the left ventricular systolic and diastolic functional parameters measured after four weeks of treatment. Aspirin treatment preserved ventricular function. The data were presented as mean±SEM; # *P* vs. TAC; * *P* vs. Control. Control, sham-operated mice treated with vehicle (PBS + 0.65% ethanol); TAC, TAC-operated mice treated with vehicle; aspirin + TAC, TAC-operated mice treated with $10\ mg \cdot kg^{-1} \cdot d^{-1}$ aspirin (human equivalent dose of $1\ mg \cdot kg^{-1} \cdot d^{-1}$); TAC, transverse aortic constriction; LVAW, left ventricular anterior wall; LVPW, left ventricular posterior wall; LVID, left ventricular interior diameter; LV Vol, left ventricular volume; d, diastolic; s, systolic.

Aspirin attenuates cardiomyocyte hypertrophy *in vitro*

Aspirin significantly reduced the ANG II-induced hypertrophic responses in NVRMs in a dose-dependent manner, as indicated by the reduced mRNA levels of β-MHC, ANP, and BNP (Fig. 3A), as well as the protein level of β-MHC (Fig. 3B). Moreover, immunohistochemical staining (Fig. 3C) demonstrated that aspirin ($100\ \mu mol \cdot L^{-1}$) diminished the cell surface area in NVRMs treated with ANG II compared with the non-treated groups. This important observation supports previous data, wherein the equivalent low aspirin concentration in human plasma has led to the almost complete inhibition of thromboxane A_2 synthesis [23,25].

Aspirin downregulates Wnt/β-catenin signaling

Evidence from recent studies on the gain-of-function mutations in conditional transgenic mice has implicated β-catenin in VH, with increased mortality upon pressure overload or chronic ANG II stimulation. Conversely, conditional deletion of the β-catenin gene significantly reduced the hypertrophic responses of animals [12]. Furthermore, Zheng and colleagues [26] reported the

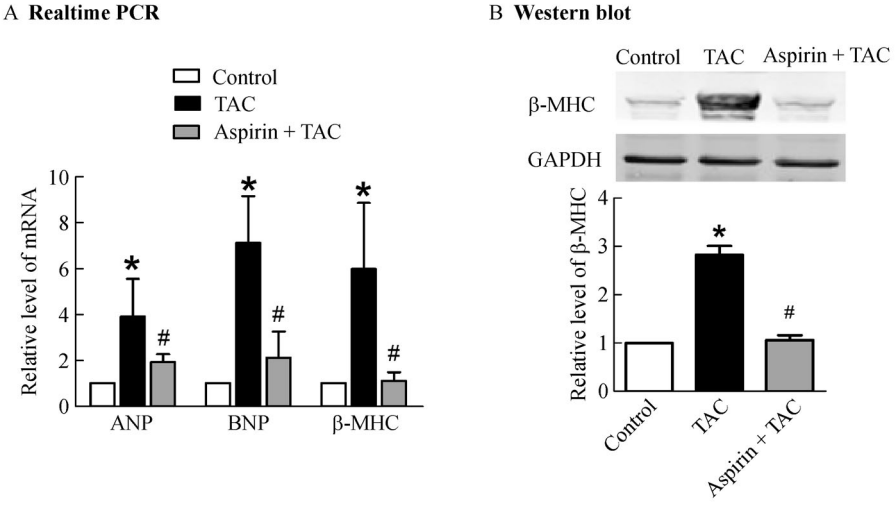

Fig. 2 Effects of aspirin on biomarkers of cardiac hypertrophy in the ventricular myocardium. (A) Real-time PCR analysis of ANP, BNP, and β-MHC mRNA. (B) A western blot analysis of β-MHC protein (upper panel) and statistical analysis (lower panel). Aspirin ($10\ mg \cdot kg^{-1} \cdot d^{-1}$) normalized the abnormal upregulation of the hypertrophic biomarker *in vivo*. The data were presented as mean ± SEM; $n = 3$; $^{\#}P < 0.05$ vs. TAC; $^{*}P < 0.05$ vs. Control. Control, sham-operated mice treated with vehicle (PBS + 0.65% ethanol); TAC, TAC-operated mice treated with vehicle; aspirin + TAC, TAC-operated mice treated with $10\ mg \cdot kg^{-1} \cdot d^{-1}$ aspirin (human equivalent dose of $1\ mg \cdot kg^{-1} \cdot d^{-1}$).

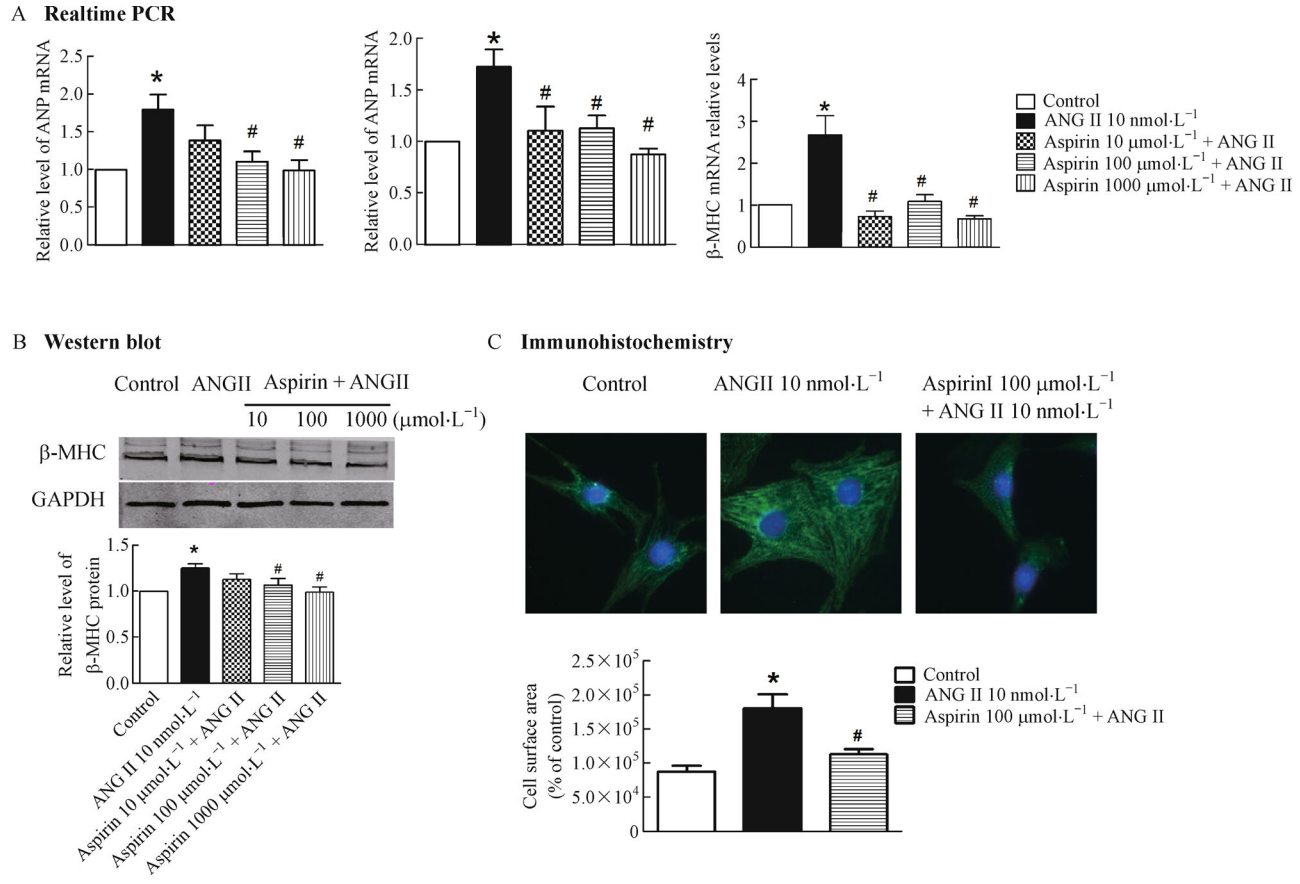

Fig. 3 Effects of aspirin on biomarkers of VH *in vitro*. (A) Real-time PCR analysis of ANP, and BNP, and β-MHC mRNA levels in ANG II (10 nmol·L^{-1}) induced the NRVM hypertrophy model. One-way ANOVA showed that aspirin normalized the abnormal upregulation of hypertrophic biomarkers in a dose-dependent manner. The data were presented as mean ± SEM; $n = 4$ for each individual experiment; $^{\#}P < 0.05$ vs. ANG II (10 nmol·L^{-1}); $^{*}P < 0.05$ vs. Control. (B) Representative western blot analysis of β-MHC protein expression (upper panel) and the corresponding statistical analysis (lower panel). The data were presented as mean ± SEM; $n = 4$ for each individual experiment; $^{\#}P < 0.05$ vs. ANG II (10 nmol·L^{-1}); $^{*}P < 0.05$ vs. Control. (C) Representative immunohistochemical staining of NRVM photographed by fluorescence microscopy (upper panel) and its statistical analysis (bottom panel). DAPI and α-actinin were used to stain the nuclei and cytoplasm, respectively. Aspirin (100 μmol·L^{-1})-treated NRVM showed reduced cell size compared to non-treated cells; 10 cells were scored per field. The data were presented as mean ± SEM; neonatal rats per individual experiment, $n = 3$; $^{\#}P < 0.05$ vs. ANG II (10 nmol·L^{-1}); $^{*}P < 0.05$ vs. Control. NRVM, primary neonatal cardiomyocytes; Control, NRVM treated with vehicle (DMSO at a final concentration of < 0.01%); ANG II (10 nmol·L^{-1}), NRVM treated with ANG II alone; Aspirin + ANG II, NRVM treated with aspirin (varying concentrations) + ANG II.

nuclear localization of β-catenin in ventricles of hypertensive rats. Aspirin reportedly inhibits Wnt/β-catenin signaling in cancer cells [9], and β-catenin expression can be activated by ANG II exposure [27]. Consequently, we hypothesized that low-dose aspirin may produce anti-hypertrophic effects through the downregulation of β-catenin expression in our models.

To test this hypothesis, we initially assessed the effects of aspirin on β-catenin expression. We found that the expression of β-catenin was markedly reduced at the mRNA and protein levels in aspirin-treated animals and NVRM (Fig. 4A and 4B). We then analyzed the relative levels of β-catenin between cytosolic and nuclear fractions and observed the significant reduction of nuclear localization after the animal tissue was treated with aspirin (Fig. 4C).

Subsequently, we evaluated the effects of the Wnt β-catenin signaling inhibitor Dickkopf-1 (Dkk1, 200 nmol·L^{-1}), which functions by directly binding to Wnt receptors LRP5/6 [12]. As shown in Fig. 5A and 5B, the pretreatment of NVRM with DKK-1 produced similar anti-hypertrophic effects, as reflected by the reduced expression of ANP, BNP, β-MHC, and β-catenin at the mRNA and protein levels. These results suggested the possibility that aspirin, at least partially, functions via the Wnt/β-catenin pathway to achieve its anti-hypertrophic effect.

Aspirin modulates Wnt/β-catenin signaling by downregulating Akt

GSK-3β (a component of the axin/APC/CK1α/GSK-3β

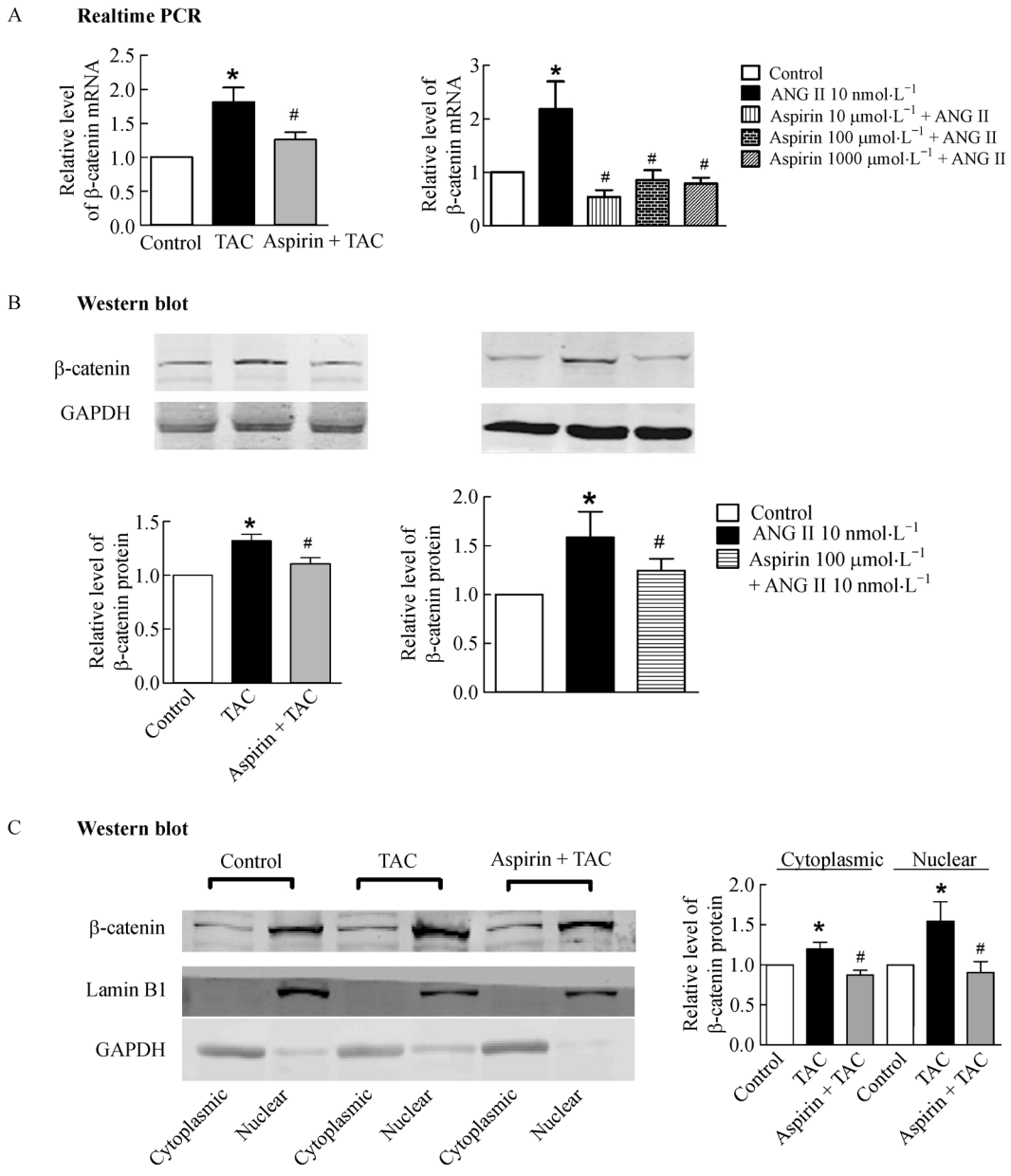

Fig. 4 Effects of aspirin on Wnt/β-catenin expression. (A) Real-time PCR analysis of β-catenin mRNA expression in cardiac tissue (left) and NVRMs (right). One-way ANOVA showed aspirin treatment ($10\ mg \cdot kg^{-1} \cdot d^{-1}$) significantly reduced β-catenin expression in a dose dependent manner. The data were presented as mean ± SEM; $n = 3$; $^{\#}P < 0.05$ vs. TAC or ANG II $10\ nmol \cdot L^{-1}$; $^{*}P < 0.05$ vs. Control. (B) Representative western blot analysis of total β-catenin protein expression (upper panel) in cardiac tissue (left) and NRVMs (right), with the corresponding statistical analysis (lower panel). Treatment significantly reduced β-catenin protein expression; $n = 3$; $^{\#}P < 0.05$ vs. TAC or ANG II $10\ nmol \cdot L^{-1}$; $^{*}P < 0.05$ vs. Control. (C) Representative western blot analysis of cytosolic and nuclear β-catenin protein expression in cardiac tissue (left) and the mean data of band density (right). The cytoplasmic and nuclear β-catenin fractions were normalized to GAPD and lamin B1, respectively. Aspirin significantly reduced the nuclear β-catenin expression in aspirin-treated and TAC-operated mice. The data were presented as mean ± SEM; $n = 3$; $^{\#}P < 0.05$ vs. TAC; $^{*}P < 0.05$ vs. Control. Control, sham-operated mice treated with vehicle (PBS + 0.65% ethanol); TAC, TAC-operated mice treated with PBS + 0.65% ethanol; Aspirin + TAC, TAC-operated mice treated with aspirin $10\ mg \cdot kg^{-1} \cdot d^{-1}$ (human equivalent dose of $1\ mg \cdot kg^{-1} \cdot d^{-1}$).

destruction complex) enhances β-catenin proteolytic degradation by proteasomes [12]. Akt (PKB) phosphorylates β-catenin near the C-terminal to increase its dissociation from the adherent junctions [28] and concomitantly phosphorylates GSK-3β (Ser^{9}) to inhibit its kinase activity [12]; thus, β-catenin transcription activity increases. NSAIDs negatively regulate Akt via a COX-independent mechanism [29]; thus, we hypothesized that low-dose aspirin may modulate Wnt signaling by down-regulating the Akt expression in our models. Our data

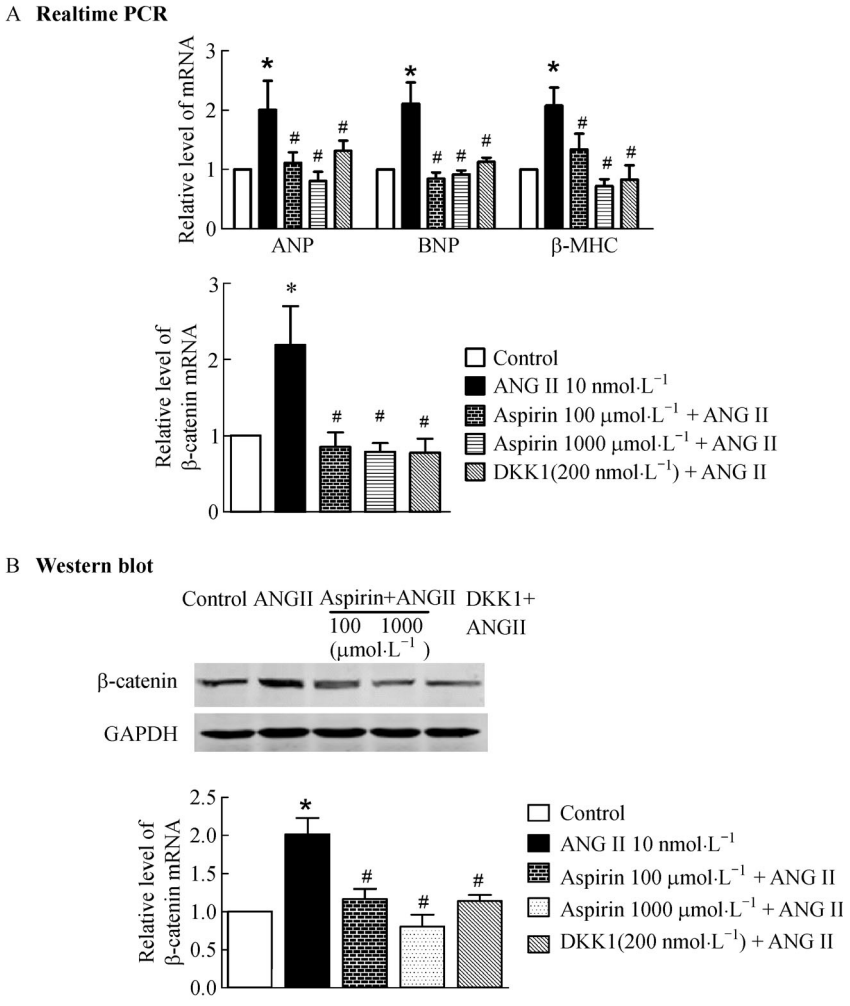

Fig. 5 Effects of aspirin and Dickkopf-1 on β-catenin and hypertrophy markers expression. (A) Real-time PCR analysis of β-catenin, ANP, BNP, and β-MHC mRNA. (B) Representative western blot analysis of total β-catenin protein expression in NRVM (upper panel) and mean data of band density (lower panel). NVRMs were incubated with or without aspirin (100 μmol·L^{-1}) or with DKK-1 (200 nmol·L^{-1}), a specific Wnt-β-catenin signaling inhibitor, in the presence or absence of ANG II (10 nmol·L^{-1}). The anti-hypertrophic effects of aspirin and DKK were similar. The data were presented as mean ± SEM, neonatal rats per individual experiment, $n = 3$, $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Control. Control, NVRM treated with DMSO to a final concentration of < 0.01%; ANG II (10 nmol·L^{-1}), NRVM treated with ANG II alone; Aspirin + ANG II, NRVM treated with aspirin (100 or 1000 μmol·L^{-1} concentrations) + ANG II; DKK + ANG II, NVRM treated with Dickkopf (200 nmol·L^{-1}) and ANG II.

supported this hypothesis, that is, aspirin treatment down-regulated the abnormally increased level of P-Akt (Ser^{473}) (Fig. 6A and 6B). Concomitantly, the level of inactive GSK-3β (Ser^{9}) was also decreased (Fig. 6C and 6D) in cardiac tissue and NVRM.

Discussion

VH is a hallmark of CVD; its direct cardiac effects include ventricular dysfunction and increased risk of cardiac events [2,3]. Most importantly, the reversal or attenuation of VH is associated with improved clinical outcomes [2,30,31]. Aspirin trials have investigated its vascular effects on the prevention or treatment of CVD [4–6,15], although the sequelae of VH resembles overt coronary diseases [2,3,32].

Our study mainly aimed to investigate the anti-hypertrophic properties of the "anti-thrombotic" dose. The results revealed notable improvement in cardiac functions, which are marked by the reduced ventricular mass and the heart weight-to-tibia length ratio.

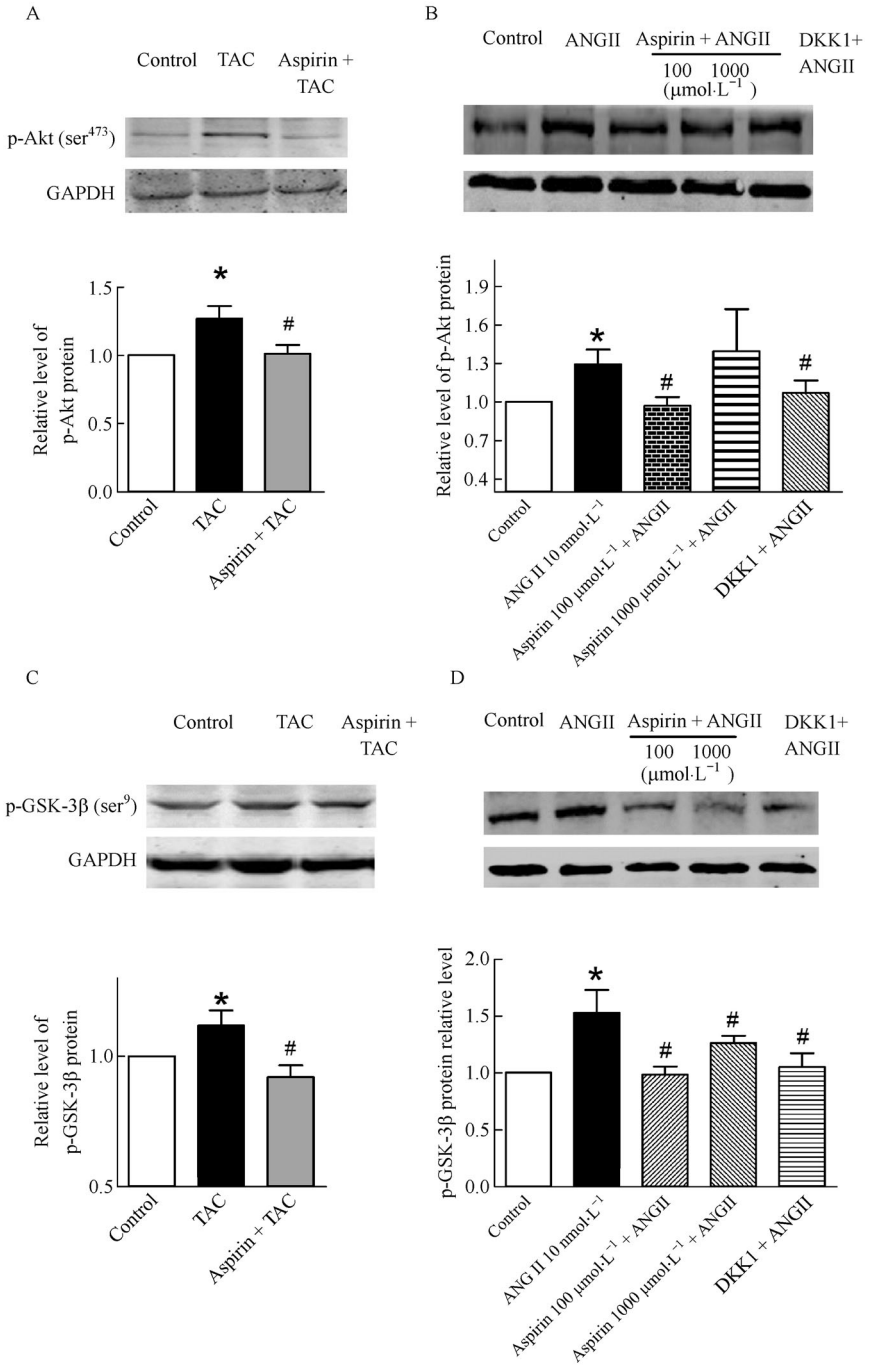

Fig. 6 Effects of aspirin on Akt and GSK-3β expression. (A) Representative western blot analysis of p-Akt (Ser^{473}) protein expression in cardiac tissue (upper panel) and the mean data of band density (lower panel). Aspirin abrogated the abnormal upregulation of p-Akt (Ser^{473}) protein expression. The data were presented as mean ± SEM, $n = 4$; $^{\#}P < 0.05$ vs. TAC, $^{*}P < 0.05$ vs. Control. (B) Representative western blot analysis of p-Akt (Ser^{473}) protein expression in NVRM treated with aspirin (100 or 1000 μmol·L^{-1}) or DKK-1 (200 nmol·L^{-1}) in the presence or absence of ANG II (10 nmol·L^{-1}; upper panel). The mean data of band density (lower panel) were presented as mean ± SEM, $n = 3$; $^{\#}P < 0.05$ vs. ANG II, $^{*}P < 0.05$ vs. Control. (C) Representative western blot analysis of the phosphorylated and inactive forms of GSK-3β (Ser^{9}) protein levels in cardiac tissue (upper panel) and the mean data of band density (lower panel). Aspirin abrogated the abnormal upregulation of GSK-3β (Ser^{9}) protein expression. The data were presented as mean ± SEM, $n = 4$; $^{\#}P < 0.05$ vs. TAC, $^{*}P < 0.05$ vs. Control. (D) Representative western blot analysis of p-GSK-3β (Ser^{9}) protein expression in NVRM treated with aspirin (100 or 1000 μmol·L^{-1}) or DKK-1 (200 nmol·L^{-1}) in the presence or absence of ANG II (10 nmol·L^{-1}; upper panel) and the mean data of band density (lower panel). The data were represented as mean ± SEM, $n = 3$; $^{\#}P < 0.05$ vs. ANG II (10 nmol·L^{-1}), $^{*}P < 0.05$ vs. Control. NVRM, primary cultured neonatal cardiomyocytes; Control, sham-operated mice treated with vehicle (PBS + 0.65% ethanol) or NVRM treated with DMSO to a final concentration of < 0.01%; TAC, TAC-operated mice treated with PBS + 0.65% ethanol; Aspirin + TAC, TAC-operated mice treated with aspirin 10 mg·kg^{-1}·d^{-1} (human equivalent dose of 1 mg·kg^{-1}·d^{-1}); ANG II (10 nmol·L^{-1}), NRVM treated with ANG II alone; Aspirin + ANG II, NRVM treated with aspirin (varying concentrations) + ANG II; DKK-1 + ANG II, NVRM treated with Dickkopf 1(200 nmol·L^{-1}) and ANG II.

Furthermore, aspirin treatment produced fewer and smaller degenerative ultrastructural alterations; aspirin treatment also concomitantly reduced the expression of the molecular markers of hypertrophy (ANP, BNP, and β-MHC). The results were observed in aspirin within its "antiplatelet" dosage range [22,23,25,33]. Our findings strongly suggested that aside from its anti-thrombotic properties, aspirin confers cardiovascular benefits via the attenuation of VH. Previous animal studies have reported protective effects at aspirin doses ($100\,mg\cdot kg^{-1}\cdot d^{-1}$) that were significantly higher than normally recommended for CVD [34,35]. The present study found a marked anti-hypertrophic effect at aspirin concentrations of 10 $\mu mol\cdot L^{-1}$. The dose and concentrations used in the present study, when corrected to human dosages, coincided with the recommendations for the prevention of cardiovascular events in the guidelines of the American College of Cardiology/American Heart Association [6] and the European Society of Cardiology [36].

In *in vivo* experiments, $75\,mg\cdot d^{-1}$ human aspirin dose was used to derive animal equivalent doses; in *in vitro* studies, C_{max} used to simulate the milieu of low aspirin concentrations in NVRM was derived from $100\,mg\cdot d^{-1}$ [23,25]. However, published pharmacokinetics studies on this aspirin dose have revealed contradictory C_{max} depending on formulation, such as soluble, rapid-release, or chewable 100 mg tablets exhibiting minor differences [22,23,33]; by contrast, the enteric or controlled release tablets showed wide and significant variations [37,38]. Our *in vitro* results simulated human plasma levels based on 100 mg rapid-acting aspirin formulations (soluble and rapid-release tablets), whose quick action is desirable during the acute onset of symptoms, such as in MI. The human plasma levels of 10 and 100 $\mu mol\cdot L^{-1}$ were approximately attained by $100\,mg\cdot d^{-1}$ aspirin; furthermore, 10 $\mu mol\cdot L^{-1}$ is within the upper scale of equivalent concentrations reported in some controlled-released enteric coated aspirin tablets or formulations [37,38] or even in lower doses of aspirin (such as 75 or $80\,mg\cdot d^{-1}$) [39]. Therefore, the aspirin dosages used in our study are within the therapeutic range with clinical implications.

The antiplatelet therapy in CVD is based on the understanding that aspirin prevents the propagation of ruptured thrombus from a fibrous cap to full vascular occlusion [6,7]. However, the "anti-thrombosis" effect does not fully account for the more pronounced effects in the heart compared with other vasculature; for example, the 2009 Antithrombotic Trialists' (ATT) Collaboration reported significant reduction in nonfatal MI, but the protective effect on vascular death or stroke is not significant [4]. Similar conclusions have been generated in another meta-analysis, which included 3 additional trials and 7105 patients to the original 6 ATT trials [5]. The secondary prevention trials have reported similar results [4]. In our model, aspirin reduced VH in a dose-dependent manner, with the greatest effect observed at 1000 $\mu mol\cdot L^{-1}$. Therefore, our findings imply the potential of aspirin in the clinical settings associated with cardiac hypertrophy. However, this potential has yet to be verified. To the best of our knowledge, the reversal or attenuation of left VH by low-dose aspirin has not been investigated as an outcome in humans or animals.

Cumulative evidence links pathological VH to the increased expression of β-catenin [12,40]. A study by Zheng *et al.* [26] implicated β-catenin in the pathophysiology of hypertension, which is the main risk factor of LVH in clinical settings [2]. Clinical and experimental studies have shown that the renin-ANG-aldosterone system (RAAS) is over-activated in cardiac hypertrophy and heart failure [31]. Interestingly, experimental hypertrophy studies in mice [41] and NVRMs [27] reported the significant induction of β-catenin protein upon treatment with ANG II, the RAAS effector. Our results suggested that β-catenin is a potential therapeutic target for CVD and cardiac hypertrophy. In this study, aspirin downregulated β-catenin expression and concomitantly reduced its nuclear export.

Cardiac Akt is documented to produce antagonistic time-dependent effects on ventricular remodeling [42], that is, short-term Akt activation by various trophic signals, such as insulin growth factor, mediates physiological hypertrophy [43]. However, adult-onset hypertrophy ultimately culminates in pathological remodeling; consequently, chronic or excessive Akt expression is often observed in VH and CVD [31,44]. Akt serves dual functions with respect to Wnt signaling by mediating β-catenin dissociation from adherent junctions [13,28] and inactivating GSK-3β. Using our TAC model with chronic pressure overload for 4 weeks and our cellular model with 48 h exposure to ANG II, we observed a significant increase in Akt expression, which is consistent with the findings of Fujishima and colleagues [41]. Most importantly, a low dose of aspirin significantly reduced the phosphorylated Akt and GSK-3β expression; thus, the Akt/GSK-3β/β-catenin pathway, which mediates dilated cardiomyopathy, cardiomyocyte hypertrophy, and fibrosis, is downregulated [45].

A limitation of the present study is that the TAC model does not mimic ischemic or coronary obstruction, although post-infarction remodeling and hypertrophy is very common in clinical settings [2]. However, the results of the present study are relevant because the inhibition of Wnt/β-catenin signaling has been shown to be beneficial for myocardial infarction [46].

Conclusions

This study demonstrated the significant anti-hypertrophic properties of aspirin at clinically relevant doses for anti-

thrombotic therapy. The significant downregulation of β-catenin and Akt probably functions as the signaling mechanism of the beneficial action of aspirin. Our findings indicated that aspirin may be considered for anti-hypertrophic therapy and prevention, in addition to its effectiveness as an anti-thrombotic agent.

Acknowledgements

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Compliance with ethics guidelines

Samuel Chege Gitau, Xuelian Li, Dandan Zhao, Zhenfeng Guo, Haihai Liang, Ming Qian, Lifang Lv, Tianshi Li, Bozhi Xu, Zhiguo Wang, Yong Zhang, Chaoqian Xu, Yanjie Lu, Zhiming Du, Hongli Shan, and Baofeng Yang have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed. This study was approved by the Institutional Animal Care and Use Committee of Harbin Medical University, China (No. HMUIRB-2008-06) and the Institute of Laboratory Animal Science of China (A5655-01), and also was in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

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