Original Articles

Apocynin ameliorates pressure overload-induced cardiac remodeling by inhibiting oxidative stress and apoptosis

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Short title: Apocynin ameliorates cardiac remodeling by inhibiting oxidative stress

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Summary

Oxidative stress plays an important role in pressure overload-induced cardiac remodeling. The purpose of this study was to determine whether apocynin, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, attenuates pressure overload-induced cardiac remodeling in rats. After abdominal aorta constriction, the surviving rats were randomly divided into four groups: sham group, abdominal aorta constriction group, apocynin group, captopril group. Left ventricular pathological changes were studied using Masson’s trichrome staining. Metalloproteinase-2 (MMP-2) levels in the left ventricle were analyzed by western blot and gelatin zymography. Oxidative stress and apoptotic index were also examined in cardiomyocytes using dihydroethidium and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), respectively. Our results showed that abdominal aorta constriction significantly caused excess collagen deposition and cardiac insult. Treatment with apocynin significantly inhibited deposition of collagen and reduced the level of MMP-2. Furthermore, apocynin also decreased the NADPH oxidase activity, reactive oxygen species production and cardiomyocyte apoptotic index. Interestingly, apocynin only inhibited NADPH oxidase activity without affecting its expression or the level of angiotension II in the left ventricle. In conclusion, apocynin reduced collagen deposition, oxidative stress, and inhibited apoptosis, ultimately ameliorating cardiac remodeling by mechanisms that are independent of the renin-angiotensin system.

Key words
Apocynin • Cardiac remodeling • Angiotension II • NADPH oxidase • Reactive
oxygen species • Apoptosis.
Introduction

Heart failure (HF) is the end stage of various kinds of cardiovascular diseases (CVD), which is a leading cause of morbidity and mortality in middle age and aged people (Greenberg et al. 2012, Al Suwaidi et al. 2012). Cardiac remodeling has been suggested to be associated with poor prognosis in patients with cardiovascular disorders (Cohn et al. 2000, Gonzalez et al. 2011). Cardiomyocyte hypertrophy, apoptosis, and subsequent fibroblast proliferation play critical roles in the process of cardiac remodeling, which ultimately accelerates cardiac fibrosis and contributes to cardiac dysfunction (Hassan et al. 2012, Mitra et al. 2013).

The renin-angiotensin system (RAS) plays an important role in the cardiac remodeling process and is involved in the pathogenesis of HF (Nagalingam et al. 2013). Growing evidence confirms that activation of the RAS leads to a large generation of reactive oxygen species (ROS) that contribute to cardiomyocyte hypertrophy, apoptosis, and fibrosis in cardiac remodeling (Choudhary et al. 2008). Captopril, as the earliest angiotensin converting enzyme inhibitor (ACEI), exerts cardioprotective effects by increasing tissue antioxidant activity, scavenging different types of ROS, and then decreasing the deposition of collagen, inflammatory response and myocyte apoptosis, which ultimately ameliorating cardiac remodeling (St John Sutton et al. 1997, Li et al. 2015). Recent studies showed that ROS are produced by mitochondria, xanthine oxidases, cytochrome P450 reductase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Bedard and Krause 2007). Among these, NADPH oxidase is a primary source for ROS production at the presentation of CVD. NADPH oxidase
contains phox units (gp91phox, p22phox, p40phox, p47phox, p67phox) and the small GTPase (Rac1 or Rac2) (Cai et al. 2003, Jin et al. 2008, Lassegue and Griendling 2010). ROS derived from NADPH oxidase play a vital role in pathophysiological conditions such as atrial fibrillation, myocardial infarction, and hypertension (Liu et al. 2012b, AL-Rasheed et al. 2013, Cheng et al. 2014). Therefore, inhibition of this enzyme may be an attractive and potential therapeutic target for the treatment of HF (Schwarzer et al. 2014).

Apocynin (4-hydroxy-3-methoxy-acetophenone), a constituent of the Himalayan herb Picrorhiza kurrooa Royle (Scrophulariaceae) has been used as an inhibitor of NADPH oxidase (Engels et al. 1992, Babior et al. 2002). Recent studies showed that apocynin can improve atrial fibrillation in hearts with infarction, and also has antihypertensive effects on spontaneously hypertensive rats (Sovari et al. 2008, Ciarcia et al. 2010). We previously reported that apocynin ameliorated pressure overload-induced cardiac hypertrophy by reducing ROS production (Liu et al. 2010). However, it is not clear whether apocynin can attenuate cardiac remodeling in rats with pressure overload. Therefore, in the present study we investigated whether apocynin might be able to attenuate cardiac remodeling in Sprague-Dawley (SD) rats with pressure overload. We also further explored the possible mechanisms of action of apocynin for suppressing cardiac fibrosis.

**Material and Methods**

*Animals*
Adult male SD rats (8-10 week-old) used in this study, were supplied by the Experimental Animal Centre of Xi’an Jiaotong University. The animals were housed under controlled temperatures (20 ± 5°C), humidity (60%-75%), and light/dark cycles (12 h day/12 h night). All experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1996). The animal protocols were approved by the Animal Care and Use Committees of Xi’an Jiaotong University.

*Induction of cardiac remodeling via abdominal aorta constriction and drug administration*

Abdominal aorta constriction was performed as described previously (Chen et al. 2010). Briefly, after being fasted for 12 h, rats (n = 50) were anesthetized with pentobarbital sodium (45 mg/kg) by intraperitoneal injection, and the aorta was dissected above the two renal arteries. A silver clip (0.70 mm internal diameter) was placed on the aorta abdominalis above the level of the left renal arteries. 7 days after the surgical procedure, rats were randomly divided into four groups: abdominal aorta constriction group (AAC, n = 7), apocynin (Apo) + AAC group (Apo, 200 mg/kg/day, n = 8), captopril (Cap) + AAC group (Cap, 75 mg/kg/day, n = 8). The Sham group (n = 10) was subjected to the same surgical procedure without clip placement. All drugs were purchased from Sigma (St Louis, MO, USA) and dissolved in drinking water. The animals were sacrificed 8 weeks after surgery and the hearts were collected for histological analysis.

*Hemodynamic data analysis*
To examine the effect of Apo on cardiac function, we measured hemodynamics 8 week after abdominal aorta constriction. Animals were anesthetized with pentobarbital sodium (45 mg/kg) by intraperitoneal injection, and the right carotid arterial connected to pressure transducers was introduced into the left ventricle to measure heart rate (HR), systolic arterial pressure (SAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and maximum/minimum values of the first derivative of left ventricular pressure (dp/dt$_{\text{max}}$ and dp/dt$_{\text{min}}$). All data were recorded with PowerLab (AD Instruments) in all groups under a 30-minute steady-state condition.

**Blood sampling and tissue preparation**

At the eighth week, all rats were anesthetized with pentobarbital sodium by intraperitoneal injection. Blood was drawn from the abdominal aorta, allowed to clot, centrifuged at 5000 × g for 10 min and the serum was stored at -80°C for further analysis. Next, the whole hearts from the rats were quickly removed, washed with cold phosphate buffered saline (2.7 mM KCl, 137 mM NaCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$ at pH 7.4), and sliced transversely from apex to base freeing the right ventricular wall. The left ventricular wall was snap-frozen in liquid nitrogen and store at -80°C. The angiotensin II (Ang II) activity (concentration) in tissue and serum was analyzed using a commercial kit (Dong Ya Radioimmunology Institute, Beijing, China) following the manufacturer’s instructions.

**Collagen deposition determination by Masson’s trichrome staining**

Masson’s trichrome staining was performed in the rat hearts to determine collagen deposition. Hearts were excised, washed in PBS at 4°C, cut into 3 transverse
sections close to the apex to visualize the left and right ventricles, and then fixed in 4% formalin by paraffin embedding. They were sectioned (5 μm) and stained. Cardiac fibrosis was analyzed by morphometry via Masson’s staining and visualized by light microscopy.

Assessment of oxidative stress

Myocardial superoxide anion production was measured using the fluorescent dye, dihydroethidium (DHE; Beyotime Institute of Biotechnology, Nanjing, China) as described previously.(Courtois et al. 2003) Briefly, 5 μm fresh frozen sections from the hearts were incubated in DHE (10 μM) for 30 min at 37°C and photographed under fluorescent microscopy. The red fluorescence intensity in sections was analyzed using the Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD).

Heart tissue was homogenized in ice cold PBS with a homogenizer to yield a 10% (wt/vol) myocardial homogenate. According to the manufacturer’s instructions, Malondialdehyde (MDA) level, superoxide dismutase (SOD) and NADPH oxidase activity (Bio-Rad Laboratories, Hercules, CA, USA) were colorimetrically analyzed using a microplate spectrophotometer at 455 nm, 500 nm, and 550 nm, respectively.

Assessment of apoptosis

To assess myocardial cell death or apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using a DNA fragmentation/fluorescence staining TUNEL apoptosis detection kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol. TUNEL positive cells were visualized by fluorescence microscopy (BX50, Olympus Co. Ltd., Tokyo, Japan) and
the TUNEL index was calculated as the ratio of all groups compared to the Sham group.

Caspase-3 activity in myocardial tissue was evaluated using a microplate spectrophotometer at 405 nm by the biochemical reagent kit (Beyotime Institute of Biotechnology, Jiangsu, China).

**Real-time PCR**

Total RNA was extracted from left ventricular tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with a phenol-chloroform single step isolation. Reverse transcription was performed with an ExScript RT reagent kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s protocol. cDNA was diluted and amplified using random primers. The primer sequences for each gene were as follows:

- p22\textsuperscript{phox}: F-ATGGAGCGGTGTGGACAGAAG and R-CGGACAGCAGTAAGTGAGGAC; p67\textsuperscript{phox}:
  - F-CCAGGCATTCCAAGATTGACAAGG and R-TGAACCACAGAGGCTACAACGC;
- β-Actin: F-CTATCGGCAATGAGCGGTTCC and R-TGTGTTGGCATAAGGGTCTTACC.

Relative RNA levels were expressed as the p22\textsuperscript{phox}/p67\textsuperscript{phox} to β-actin ratio.

**Western blotting**

To examine the effects of apocynin on cardiac remodeling, levels of matrix metalloproteinase-2 (MMP-2), p22\textsuperscript{phox} and p67\textsuperscript{phox} were measured. Samples (30 μg) were loaded onto 10% gradient SDS gels, separated by electrophoresis, and subsequently electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% BSA in Tris-buffered saline
containing 0.1% Tween (TBST), the membrane was incubated with the following primary antibodies; MMP-2 (diluted 1:1000, Bioworld technology Co., Ltd., Nanjing, China), p22\textsuperscript{phox} (diluted 1:1000, Cell Signaling Technology, Co., Ltd., Columbia, US), and p67\textsuperscript{phox} (diluted 1:1000, Cell Signaling Technology, Co, Ltd., Columbia, US) overnight at 4°C. Protein bands were detected using an ECL-Plus kit (PerkinElmer Life Science, Waltham, MA, USA) and quantified by densitometry using Quantity One software (Bio-Rad Laboratories, Berkeley, CA).

**Gelatin zymography**

Samples in a solution of 10% SDS, 20% glycerol, 0.5% bromophenol blue, and 0.125 mM Tris-HCl (pH 6.8) were loaded on 0.1% gelatin zymogram gels. Separating gels were washed twice for 30 min in 100 mL of Triton X-100 (2.5%) and incubated overnight at 37 °C in a developing buffer containing 50 mM Tris, 5 mM CaCl\textsubscript{2}\cdot H\textsubscript{2}O, 0.2 mM NaCl, and 0.02% polyethylene glycol monododecyl ether (Brij-35; Tokyo Chemical Industry, Co, Ltd, Tokyo, Japan). Gels were stained for 30 min in 0.5% Coomassie blue R-250, 10% glacial acetic acid, and 10% isopropanol and de-stained in 50% methanol acid and 10% acetic acid for 10 min, then dried and scanned.

**Statistical analysis**

Data are presented as mean ± SEM. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Turkey’s multiple comparison test using GraphPad Prism Version 5.01 (GraphPad Software, Inc., La Jolla, CA). Statistical significance was defined as a value of \( p < 0.05 \).
Results

Effects of Apo on hemodynamics

Hemodynamic parameters were measured at the end of the experiments. Our data showed that there was a significant difference in HR in AAC group as compared to the Sham group (Figure 2A; *p* < 0.05). Neither captopril nor Apo treatment influenced HR (Figure 2A). SAP was significantly increased in AAC group (Figure 2B, *p* < 0.01), and treatment with captopril but not apocynin reduced SAP in Cap group compared with AAC (Figure 2B; *p* < 0.05). Both LVSP and LVEDP were significantly increased in AAC group compared with Sham (Figure 2C; *p* < 0.01). Captopril administration significantly reduced LVSP and LVEDP (Figure 2C, D; *p* < 0.01). Treatment with Apo only reduced LVEDP in Apo group (Figure 2D; *p* < 0.05). Characteristic impairments in dp/dt\(_{\text{max}}\) and dp/dt\(_{\text{min}}\) were markedly increased in AAC (Figure 2E, F; *p* < 0.01). and only Apo markedly reduced dp/dt\(_{\text{min}}\) in Apo group (Figure 2F; *p* < 0.05).

Effects of apocynin on interstitial fibrosis

To evaluate interstitial fibrosis in heart tissue, collagen deposition was measured by Masson’s trichrome staining. Compared with the Sham group, the interstitial fibrosis was significantly increased in AAC group. However, this increase was remarkably lower in the Cap and Apo groups (Figure 3B, *p* < 0.01).

Effects of apocynin on expression of Ang II in serum and cardiac tissue

The effects of apocynin and captopril on concentration of Ang II were assessed and results showed no significant difference in expression levels in the serum of all groups (Figure 4A). However, the Ang II levels increased in cardiac tissue from the
AAC group compared with the Sham group (Figure 4B, \( p < 0.01 \)). Treatment with captopril but not apocynin had a significant effect on the levels of Ang II in cardiac tissue (Figure 4B, \( p < 0.01 \)).

*Effects of apocynin on NADPH oxidase*

There was a significant increase in NADPH oxidase activity in the AAC group compared with the Sham group; however, its activity decreased in the presence of captopril and apocynin (Figure 5A, \( p < 0.01 \)). Analysis of NADPH oxidase expression by western blot and RT-PCR demonstrated a significant increase in both protein and mRNA levels, respectively, for \( p22^{phox} \) and \( p67^{phox} \) in the AAC group compared to the Sham group (Figure 5B-F, \( p < 0.05 \)). Treatment with captopril but not apocynin for 8 weeks had a significant effect on the levels of \( p22^{phox} \) and \( p67^{phox} \) (Figure 5B-F, \( p < 0.05 \)).

*Effects of apocynin on ROS*

Superoxide anion levels were examined in cardiomyocytes using DHE staining. Our results showed that generation of superoxide anion was significantly elevated in cardiomyocytes from the AAC group compared with the Sham group (Figure 6A, B, \( p < 0.01 \)). These changes were significantly reversed by captopril or apocynin administration (Figure 6A, B, \( p < 0.05 \)). We further examined the effects of captopril and apocynin on MDA and SOD. The concentration of MDA was significantly higher in the AAC group compared with the Sham group (Figure 6C, \( p < 0.01 \)). Treatment with captopril and apocynin significantly reduced the level of MDA (Figure 6C, \( p < 0.01 \)). Conversely, the activity of SOD was significantly decreased in the AAC group.
compared with the Sham group. Treatment with captopril or apocynin increased SOD activity significantly, in the myocardium (Figure 6D, \( p < 0.05 \)).

**Apocynin reduced TUNEL staining and the expression of active caspase-3 in cardiomyocytes**

To study the effects of apocynin on cardiac apoptosis in myocytes, we evaluated the apoptotic index. TUNEL assay indicated that there was a significant increase in cardiomyocyte apoptosis in the AAC group (Figure 7A, B, \( p < 0.01 \)). Treatment with captopril or apocynin significantly attenuated apoptosis compared with the AAC group (Figure 7B, \( p < 0.01 \)). In line with the TUNEL staining, activity of caspase-3 was significantly increased in the AAC group compared with the Sham group (Figure 7C, \( p < 0.01 \)). Moreover, administration of captopril or apocynin significantly reduced the activity of caspase-3 (Figure 7C, \( p < 0.01 \)).

**Effects of apocynin on MMP-2**

MMP-2, a gelatinase that is crucial for degrading the extracellular matrix, has been implicated as an important factor in cardiac remodeling. We therefore investigated the expression and activity of MMP-2 in cardiac tissue using western blot and gelatin zymography. As shown in Figure 8, the expression and activity of MMP-2 was significantly increased in the AAC group compared with the Sham group. Treatment with captopril or apocynin significantly reduced the expression and activity of MMP-2 in cardiac tissue (Figure 8A-D, \( p < 0.01 \)).

**Discussion**
In the present study, we examined the effects of apocynin on cardiac remodeling induced by pressure overload. We found that apocynin, the NADPH oxidase inhibitor, could attenuate cardiac remodeling by reducing the deposition of collagen, reducing the level of MMP-2 and by inhibiting up-regulated NADPH oxidase activity without affecting the expression of NADPH oxidase (p22^{phox} and p67^{phox}). Additionally, apocynin ameliorated apoptosis in cardiomyocyte by suppressing the excessive production of ROS. Our results indicate that apocynin has cardioprotective effects, which might be related to its antioxidative effects.

Cardiac remodeling secondary to pressure overload is characterized by inappropriate hypertrophy, fibrosis, and apoptosis (Rain et al. 2014). RAS is a key regulator of pathophysiological processes and plays pivotal roles in cardiac remodeling, by accelerating fetal gene expression and collagen deposition. Ang II, a central biologically active downstream peptide of RAS, is directly involved in NADPH oxidase activation, oxidative stress, and cell apoptosis in myocardium during the pathogenesis of cardiac insult, and promotes unfavorable remodeling (Siddesha et al. 2013, Sumitomo-Ueda et al. 2010). Therefore, inhibiting the level of Ang II or ROS may as a pharmacological therapy to decrease cardiac remodeling and mortality in patients with HF. Reduction the level of Ang II by captopril, an ACEI, is used widely and highly effective in the treatment of HF through suppressing the activation RAS in clinical trials (Yoo et al. 2015). In addition, more and more studies from animal experiment further revealed that one of the protective mechanisms of captopril on CVD was closely related to the reduction of ROS (Kim et al. 2013, Kojsova et al. 2006). Apocynin is extracted
from the root of *Picrorhiza kurroa*, which has been used as an efficient inhibitor of NADPH oxidase in many experimental models involving phagocytic and nonphagocytic cells. Apocynin has multiple protective effects on a variety of organs and tissues without interfering with other aspects of immune system (Chiang et al. 2011, Li et al. 2013, Li et al. 2012). Li et al. showed that apocynin suppresses myocardial oxidative stress and produces cardioprotective effects in Ang II-induced cardiac diastolic dysfunction in mice, which might be mediated partly through a pathway involving NADPH oxidase and osteopontin proteins (Li et al. 2013). Our data showed that both apocynin and captopril ameliorate cardiac remodeling (Figure 3). During this process, the level of Ang II in the left ventricle was reduced by captopril, but not apocynin (Figure 4B). Additionally, the present study found no differences in serum Ang II levels in all groups (Figure 4A). These results indicate that apocynin could attenuate cardiac remodeling independent of RAS.

It is well known that ROS derived from NADPH oxidase plays a critical role in the development and progression of cardiac remodeling (Yang et al. 2013, Taye et al. 2013). The inhibition of NADPH oxidase or reduction of superoxide anion generation represents an effective treatment for cardiac remodeling by reducing oxidative stress (Bai et al. 2013, Niu et al. 2012). The present study demonstrated that apocynin treatment could significantly inhibit the activity of NADPH oxidase (Figure 5), reducing the superoxide anion production in the rat heart (Figure 5A), and attenuating cardiac remodeling. These improvements were associated with a significant increase in SOD activity and decrease in MDA concentration (Figure 6C, D). Therefore, these
results further support that apocynin has obvious protective effect against cardiac remodeling induced by pressure overload, which might be related to its antioxidative effects.

A noticeable increase in cardiomyocyte apoptosis and a disproportionate accumulation of collagen, as well as MMPs in extracellular matrix were observed in rodents with pressure overload (Reddy et al. 2013). Furthermore, apoptosis resulting from oxidative stress plays an important role in cardiac remodeling, and inhibition or attenuation of cardiomyocyte apoptosis could prevent cardiac remodeling (Hill et al. 2013). Our findings suggest that apocynin provides significant antioxidative and anti-apoptotic effects (Figure 7). These results imply that the beneficial effect of attenuating cardiomyocyte apoptosis mediated by apocynin might be related to inhibition of oxidative stress. MMPs have been viewed as potential and promising targets in CVD because of their key function as regulators of interstitial changes that degrade the collagen matrix (Brower et al. 2007). MMP-2, a member of the zinc-dependent proteinases, plays an important role in cardiac fibrosis (Molina et al. 2009). Some studies showed that MMP-2 was increased by NADPH oxidase overexpression at the mRNA, protein, and activity levels implying that, decreased oxidative stress and down-regulation of MMP-2 might prevent left ventricular structural remodeling (Hayashidani et al. 2002, Ceron et al. 2010, Liu et al. 2012a). In the present study, we found that apocynin significantly reduced MMP-2 levels in the AAC group and attenuated cardiac remodeling (Figure 8). These results suggest that the effect of apocynin on MMP-2 may be related to its antioxidative effect.
Compared with captopril, apocynin plays an important role in inhibition the generation of ROS by suppressing directly the activity of NADPH oxidase without effect on the level of Ang II (Engels et al. 1992, Babior et al. 2002). The effect of apocynin on inhibition of ROS may bring us a new perspective for the treatment of cardiac remodeling.

In summary, the present study confirmed that apocynin reduced the production of ROS derived from NADPH oxidase, and inhibited disproportionate accumulation of collagen as well as cardiomyocyte apoptosis. Apocynin, however, had no effects on the level of Ang II in the left ventricular wall, which is involved in cardiac remodeling induced by pressure overload. Thus, apocynin markedly ameliorated cardiac remodeling, but this cardioprotective effect was independent of the RAS. The effect of apocynin on cardiac remodeling may form the basis for its use as therapeutic in CVD, and provide valuable information for CVD prevention and treatment.

**Conflict of Interest**

There is no conflict of interest.

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**Figure legends**

![Figure diagram](image)

**Fig.1.** The experimental groups used in the study. Rats (n = 40) were anesthetized with pentobarbital sodium (45 mg/kg) by intraperitoneal injection, and a silver clip (0.70 mm internal diameter) was placed on the aorta abdominalis above the level of the left renal arteries. 7 days after the surgical procedure, rats were randomly divided into four groups: Sham, rats without abdominal aorta constriction; AAC, rats with abdominal aorta constriction; Apo, AAC rats treated with apocynin; Cap, AAC rats treated with captopril. The animals were sacrificed 8 weeks after surgery and the hearts were collected for histological analysis.
Fig. 2. Hemodynamic assessment of cardiac function after apocynin treatment in AAC group induced by pressure overload. Animals were anesthetized with pentobarbital sodium by intraperitoneal injection, and the right carotid arterial connected to pressure transducers was introduced into the left ventricle to measure HR (A), SAP (B), LVSP (C), LVEDP (D), dp/dt\(_{\text{max}}\) (E) and dp/dt\(_{\text{min}}\) (F) at 8 weeks after abdominal aorta constriction. Data are presented as mean ± SEM, **p < 0.01 compared with Sham group, ##p < 0.01 compared with AAC group. HR: heart rate, SAP: systolic arterial pressure, LVSP: left ventricular systolic pressure, LVEDP: left ventricular end-diastolic pressure, dp/dt\(_{\text{max}}\) and dp/dt\(_{\text{min}}\): maximum/minimum values of the first derivative of left ventricular pressure.
Fig. 3. Effects of apocynin on cardiac fibrosis induced by pressure overload in AAC group. (A) Representative collagen expression staining with Masson’s trichrome and cardiac fibrosis was analyzed by morphometry via Masson’s staining and visualized by light microscopy, scale bar = 200μm. (B) Statistic results of myocardial fibrosis. Data are presented as mean ± SEM. **$p < 0.01$ compared with Sham group, ##$p < 0.01$ compared with AAC group.
**Fig. 4.** Effects of apocynin on the levels of Ang II in serum and cardiac tissue. The level of Ang II in serum and cardiac tissue was measured by Radioimmunoassay. (A) Effect of apocynin on Ang II in serum. (B) Effect of apocynin on Ang II content assay in left ventricles. Data are presented as mean ± SEM, **p < 0.01** compared with Sham group, ***p < 0.01*** compared with AAC group.
Fig. 5. Apocynin attenuates activity of NADPH oxidase in rats with pressure overload. (A) The activity of NADPH oxidase in left ventricle tissue were measured by colorimetrically analyzed. (B) Representative protein expression of p22phox and p67phox in left ventricles. (C, D) Statistic results of p22phox and p67phox (E, F) The relative RNA expression level of p22phox and p67phox in left ventricles from rats were prepared and analyzed by RT-PCR. Data are presented as mean ± SEM, *p < 0.05 and **p < 0.01 compared with Sham group, #p < 0.05 and ##p < 0.01 compared with AAC group.
Fig. 6. Apocynin inhibits oxidative stress induced by pressure overload. (A) Representative images under fluorescence microscope, scale bar = 40μm. (B) Statistic results of confocal microscope analysis of ROS level in left ventricles. (C, D) Activities of MDA and SOD in cardiac tissue of rats were colorimetrically analyzed using a microplate spectrophotometer and displayed. Data are presented as mean ± SEM, **p < 0.01 compared with Sham group, #p < 0.05 and ##p < 0.01 compared with AAC group. ROS: reactive oxygen species, MDA: Malondialdehyde, SOD: superoxide dismutase.
**Fig. 7.** Effects of apocynin on apoptotic index induced by pressure overload. (A) Representative TUNEL-positive left ventricular cardiomyocyte nuclei (green fluorescence, arrows) were counted under a fluorescent microscope, scale bar = 150μm. (B) Quantitative result of TUNEL assay was analyzed in heart from all rats. (C) The level of caspase-3 in myocardial tissue was evaluated using a microplate spectrophotometer at 405 nm by the biochemical reagent kit. Data are presented as mean ± SEM, **p < 0.01 compared with Sham group, #p < 0.05 and ##p < 0.01 compared with AAC group.
**Fig. 8.** Apocynin inhibits expression of MMP-2 in rats with pressure overload. The expression level of MMP-2 was assayed by Western blot (A, B) and gelatin zymography (C, D). Data are presented as mean ± SEM, **p < 0.01 compared with Sham group, ##p < 0.01 compared with AAC group. MMP-2: Metalloproteinase-2.**