Protection of CAPE-\(p\)NO\(_2\) Against Chronic Myocardial Ischemia by the TGF-B1/Galectin-3 Pathway in \textit{Vivo} and in \textit{Vitro}

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Research Article

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Abstract

**Background** Although it is known that caffeic acid phenethyl ester (CAPE) and its derivatives could ameliorate acute myocardial injury, their effects on chronic myocardial ischemia (CMI) were not reported. This study aimed to investigate the potential effect of caffeic acid $p$-nitro phenethyl ester (CAPE-$p$NO$_2$, a derivative of CAPE) on CMI and underlying mechanisms.

**Methods** SD rats were subjected to high-fat-cholesterol-diet (HFCD) and vitamin D$_3$, and the H9c2 cells were treated with LPS to establish CMI model, followed by the respective treatment with saline, CAPE or CAPE-$p$NO$_2$.

**Results** *In vivo*, CAPE-$p$NO$_2$ could reduce serum lipid levels, and improve impaired cardiac function and morphological changes. Data of related assays indicated that CAPE-$p$NO$_2$ down-regulated the expression of transforming growth factor-$\beta$1 (TGF-$\beta$1) and galectin-3 (Gal-3). Besides, CAPE-$p$NO$_2$ decreased collagen deposition, the number of apoptotic cardiomyocytes and some related downstream proteins of Gal-3 in the CMI rats. Interestingly, the effects of CAPE-$p$NO$_2$ on TGF-$\beta$1, Gal-3 and other proteins expression in lung were consistent with that in heart. *In vitro*, CAPE-$p$NO$_2$ could attenuate the fibrosis, apoptosis and inflammation by activating TGF-$\beta$1/Gal-3 pathway in LPS-induced H9c2 cell. However, CAPE-$p$NO$_2$-mediated cardioprotection can be eliminated when treated with modified citrus pectin (MCP, an inhibitor of Gal-3). And in comparison, CAPE-$p$NO$_2$ presented stronger effects than CAPE.

**Conclusion** This study indicates that CAPE-$p$NO$_2$ may ameliorate CMI by suppressing fibrosis, inflammation and apoptosis via the TGF-$\beta$1/Gal-3 pathway *in vivo* and *in vitro*.

1 Introduction

Myocardial ischemia (MI) refers to a pathological state of decreased blood perfusion of heart, which results in aberrant vascular regeneration in ischemic areas, fibrosis, oxidative stress, inflammation and even cardiovascular dysfunction [1, 2], and its common cause is coronary atherosclerosis [3]. Among all MI cases, chronic myocardial ischemia (CMI) is more general in clinic, and its prevalence is increasing even in young people in recent years [4].

Transforming growth factor-$\beta$1 (TGF-$\beta$1) plays a role in cardiac remodeling and affects cell apoptosis, fibrosis and hypertrophy, which aggravates myocardial ischemia/reperfusion injury (MIRI) [5]. Experiments have confirmed that the down-regulated TGF-$\beta$1 has protective effects on myocardial apoptosis and MIRI. For example, Notoginsenoside R1 and mangiferin preconditioning could protect the heart from ischemia reperfusion-induced myocardial injury by inhibiting activation of TGF-$\beta$1-TAK1 signaling pathway [6, 7]. Moreover, TGF-$\beta$1 receptor inhibitor like SB431542 could reduce apoptosis of cardiomyocytes induced under high glucose and hypoxia/reoxygenation conditions [8].
Galectin-3 (Gal-3) is related to cancer, inflammation and fibrosis of various organs, which also mediates cell apoptosis and influences angiogenesis of ischemic area [9, 10]. Gal-3 is often associated with fibrosis and inflammation in heart disease [11, 12], and it is used as a new marker for predicting and diagnosing cardiovascular diseases [13, 14]. It was reported that elevated Gal-3 level was associated with mortality risk in people with acute decompensated heart failure [15]. It is also a novel biomarker for right ventricle remodeling in pulmonary arterial hypertension [16]. For example, Gal-3 up-regulates idiopathic pulmonary fibrosis in patients with pulmonary disease, and also participates in inflammation, proliferation and migration of lung cancer cells [17, 18].

Caffeic acid phenethyl ester (CAPE), a bioactive component from propolis, has been reported to have protective effect on the injured H9c2 cell, and could reduce the death of cardiomyocyte [19, 20]. Our group has designed and synthesized caffeic acid p-nitro phenethyl ester (CAPE-pNO2) and caffeic acid o-nitro phenethyl ester (CAPE-oNO2) as two derivatives of CAPE. These two derivatives have better protective effects on mice with diabetic cardiomyopathy, and they are also effective on heart of rats with MIRI [21, 22, 23].

As described above, TGF-β1 and Gal-3 are well-known markers of vascular fibrosis and atherosclerosis [24], but studies between Gal-3 and CMI are relatively rare. This study aims to investigate whether Gal-3 is connected with pathogenesis of CMI, whether CAPE-pNO2 has cardioprotection effect on CMI in vivo and in vitro, and whether the effect is corresponding with Gal-3, TGF-β1 and its related proteins.

2 Materials And Methods

CAPE was purchased from Meilun Biotechnology (Dalian, China), CAPE-pNO2 was synthesized, characterized and detected as described in previous study [25] (purity > 99.0%). Vitamin D3 and lipopolysaccharide (LPS) were purchased from Mairuida Technology (Beijing, China). Modified citrus pectin (MCP), penicillin, streptomycin and DMSO were purchased from Sigma Chemicals (St. Louis, MO, USA). The assay kits of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), alpha-hydroxybutyrate dehydrogenase (HBDH), lactate dehydrogenase (LDH), creatine kinase (CK) and creatine kinase isoenzymes (CK-MB) were purchased from Maccura Biotechnology (Chengdu, China). BCA protein assay kit, RIPA lysis buffer and Rat galectin-3 ELISA assay kit was purchased from Nanjing Jiancheng Biotechnology (Nanjing, China). Acridine orange (AO) were from Solarbio Biotechnology (Shanghai, China). Western Bright ECL (ECL) kit was obtained from Advansta (USA). H9c2 cardiomyocytes were obtained from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Antibodies against galectin-3 (Gal-3), B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), caspase 3, nuclear factor kappa-B (NF-κB), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), transforming growth factor β1 (TGF-β1), mothers against decapentaplegic homolog 2 (Smad2), collagen I (Col-I), collagen III (Col-III), matrix metalloproteinase-9 (MMP-9), phosphatidylinositol 3-kinase (PI3K), β-actin, horseradish
peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody and Cy3-conjugated goat anti-rabbit IgG(H + L) were purchased from Proteintech Group Inc (Wuhan, China).

2.1 Preparation of CMI rats model

Animal experiments were approved and carried out in accordance with the Animal Ethics Community of China. Male Sprague-Dawley rats, which weighed 200 ± 20 g, were purchased from the Experimental Animal Center of Chongqing Medical University [SCXK (Yu) 2017-002]. The animals were housed in a 12 h light/dark cycle and 22°C conditions, which were free access to food and water.

Rats were randomly divided into two groups: the control group (normal diet) and the CMI model group. The CMI model was established by high-fat-cholesterol-diet [26] (HFCD, containing 17.04 kJ/g total energy, consisting of 16.3% protein, 16.1% fat, 46.1% carbohydrate, 2.75% cholesterol, was purchased from Botai Hongda Biotechnology Ltd., Beijing) as well as induced by intraperitoneal injection of vitamin D₃ (2000 U/kg/d, dissolved in corn oil) for 3 consecutive days. The control group was injected with the same volume of corn oil.

After two months feeding, all rats’ serum samples were collected. Serum total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) level in rats were measured by commercially available kits as the respective manufacturer’s protocols. Besides, three rats in the model group and two rats in the control group were randomly selected to measure their two-lead electrocardiogram (ECG). After measurement, the selected rats were euthanized, and the aortas were removed and used for oil red O staining. Compared with the control, the elevation of ST segment in ECG, increase of serum TC, TG and LDL, decrease of HDL, and obvious lipid deposition in aorta were regarded as successful models.

The model rats were randomly divided into four groups (6 rats in each group): 1) the CMI (model) group treated with saline, 2) the CAPE group treated with CAPE (1 mg/kg/day), 3) the High-pNO₂ group treated with CAPE-pNO₂ (1 mg/kg/day) and 4) the Low-pNO₂ group treated with CAPE-pNO₂ (0.7 mg/kg/day), and the normal diet group remained as control group (treated with normal saline). CAPE and CAPE-pNO₂ were dissolved in 0.01% DMSO and intraperitoneally injected for 4 weeks. ECG was measured and the blood samples were collected at the end of the experiment, then the rats were euthanized. The entire heart and lung of rats were collected for the following experiments.

2.2 Detection of lipid, cardiac marker enzymes and Gal-3 in serum

Serum samples were obtained from blood samples by centrifugation (3000 rpm, 15 min, 4°C). Levels of serum lipid (TC, TG, HDL and LDL) and Cardiac marker enzyme (including HBDB, LDH, CK, and CK-MB) were measured by commercially available kits as the respective manufacturer's protocols. Gal-3 level in serum was measured by rat galectin-3 ELISA assay kit according to the instructions.
2.3 HE, Sirius red, TUNEL and immunohistochemistry staining of heart tissues

Heart tissues were fixed in 4% paraformaldehyde solution for 48 hours and embedded in paraffin to prepare 5 µm slices. After dewaxing and rehydration, slices were stained with hematoxylin-eosin (HE) and Sirius red (SR), respectively. Optical microscopy and digital camera were used to observe and photograph the images of the dyed parts.

As for TUNEL staining, after dewaxing and rehydration, the sections were treated with protease K solution fixed and sealed in turn, and washed with PBS three times after each step. Subsequently, TdT enzyme reaction solution was added for labeling, 0.3% H$_2$O$_2$/PBS was added for blocking endogenous peroxidase activity, and DAB solution was used for color rendering. TUNEL positive cells were observed by optical microscopy and photographed by digital camera.

For immunohistochemistry experiments, the effect of endogenous peroxidase was eliminated by adding 0.3% hydrogen peroxide methanol solution after dewaxing and rehydration of paraffin slices. Then the slices were incubated overnight with primary antibodies against TGF-β1, IL-6 and Gal-3 (1:100) at 4°C, respectively, and incubated with goat anti-rabbit IgG antibody conjugated with HRP for 2 hours. Then, the sections were re-dyed with DAB and hematoxylin. The slices were observed under an optical microscope and photographed with a digital camera. All the stained areas were measured and quantified by IPP.

2.4 Cell culture and preparation of CMI cell model

H9c2 cells were cultured in DMEM high-glucose medium [27] with 10% FBS and 1% penicillin-streptomycin solution in a humidified incubator with 5% CO$_2$ at 37°C.

CMI cell model was established by stimulating H9c2 cells with LPS [27, 28, 29]. H9c2 cells were divided into five groups: 1) the control group cultured for 24 h, 2) the model group cultured for 12 h, then with medium containing 1 µg/mL LPS for 12 h [30], 3) the CAPE group cultured with medium containing 20 µM CAPE for 12 h, then with medium containing 1 µg/mL LPS for 12 h, 4) the CAPE-$p$NO$_2$ group cultured with medium containing 20 µM CAPE-$p$NO$_2$ for 12 h, then with medium containing 1 µg/mL LPS for 12 h, and 5) the MCP-$p$NO$_2$ group cultured with medium containing 20 µM CAPE-$p$NO$_2$ and 5 mg/mL MCP for 12 h, then with medium containing 1 µg/mL LPS for 12 h.

2.5 Immunofluorescence

H9c2 cells were seeded into 96-well plates at a density of $2\times10^3$-$2\times10^4$, and treated as previous described. Then H9c2 cells were fixed with 4% paraformaldehyde for 15 min, washed with PBST 3 times, and permeated with 0.25% Triton-100 in PBST. Following the H9c2 cells were blocked with 1% bovine serum albumin, and then incubated with primary antibodies against TGF-β1, Gal-3, Col-I, Col-III, TNF-α and IL-6 (1:100) overnight at 4°C. Washed again, H9c2 cells were incubated with Cy3-conjugated secondary
antibodies (1:50) for 1.5 h in dark, and then stained by DAPI for 5 min. Images were captured under a fluorescence microscope and analyzed by IPP.

2.6 AO staining

H9c2 cells were planted into 6-well plates at a density of 1×10^6, and treated as previous described. H9c2 cells were incubated with AO dye solution (100 µg/mL) for 15 min in dark, then the apoptosis cells were observed using a fluorescence microscope.

2.7 Western blot assay

Heart tissues were lysed on ice with RIPA lysis buffer for 5 min to obtain homogenates. The obtained homogenates and H9c2 cells were decomposed in RIPA lysis solution on ice for 30 minutes, and then centrifuged at 4°C for 10 minutes at 12000 rpm to obtain supernatant for subsequent experiments. The total protein concentration in the supernatant was determined by BCA protein analysis kit and operated strictly according to the instructions. The protein samples were separated on SDS-PAGE gels and transferred to PVDF membranes, then the membranes were blocked in 5% non-fat dry milk resolved in PBST for 1.5 h. Subsequently, the blots were incubated with primary antibodies against Gal-3, MMP-9, Col-I, Col-III, TGF-β1, Bcl-2, Bax, TNF-α, IL-6, caspase 3, smad 2, NF-κB, IL-1β, PI3K and β-actin (1:1000) over night at 4°C. After washing with PBST, the blots were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) for 1.5 h. Washed again, the bound antibodies on the blots were visualized with enhanced chemiluminescence (ECL) reagent and quantified by Quantity One.

2.8 Statistical analysis

All experiments were performed at least in triplicate. Data were analyzed by SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and are presented as the mean value ± SD. Statistical analysis of data was carried out by single factor analysis of variance (ANOVA) and post-event multiple comparisons. p < 0.05 was considered of statistical significance.

3 Results

3.1 Effects on body weight and organ index in the CMI rats

During the experimental period, the body weight of the control was always heavier than the other groups (p < 0.05). After CAPE or CAPE-pNO_2 treatment, the body weight of CMI model rats increased significantly (p < 0.05), and the increment of body weight in the high-pNO_2 group was higher than that of CAPE group (p < 0.01) (Fig. 1a).

The heart index of the model group was lower than the control, which increased after CAPE or CAPE-pNO_2 treatment, but there was no statistical significance (p = 0.320). The lung index of the model group increased significantly compared to the control, and decreased after CAPE or CAPE-pNO_2 treatment (p < 0.01) (Fig. 1b and c).
3.2 Effects on serum lipid levels in the CMI rats

As shown in Fig. 2, the levels of TC, TG and LDL in serum of CMI rats were significantly higher \((p < 0.01)\), then decreased after CAPE or CAPE-\(p\)NO\(_2\) treatment \((p < 0.05)\). The effect of High-\(p\)NO\(_2\) was flat or better than that of CAPE, and the treatment effect of Low-\(p\)NO\(_2\) was equal to or weaker (Fig. 2a, b and d). As for HDL, the level in the model group was significantly decreased compared to the control \((p < 0.01)\), treating with CAPE-\(p\)NO\(_2\) decreased HDL level much stronger than CAPE did \((p < 0.05)\) (Fig. 2c).

3.3 Effects on changes of ECG and cardiac structure in the CMI rats

The model rats had significantly higher heart rate, and the ST segment of the Lead II ECG elevated. The positive charge of QRS wave group increased, the negative charge decreased, and the U wave charge increased. After treatment of CAPE or CAPE-\(p\)NO\(_2\), the heart rate became slower, the positive and negative charge of QRS wave group tended to be balanced, and the charge of U wave decreased. The above effects of High-\(p\)NO\(_2\) were better than CAPE (Fig. 3a).

As illustrated in Fig. 3, the levels of HBDH, LDH and CK-MB were all promoted in the model rats \((p < 0.01)\), and were also decreased with the treatment of CAPE or CAPE-\(p\)NO\(_2\) \((p < 0.05)\). The effects of CAPE-\(p\)NO\(_2\) were more remarkable than CAPE even in the lower dose (Fig. 3b, c and e). The level of serum CK had the same trend as the others, though there was no significant statistical difference (Fig. 3d).

The HE staining of the heart was shown in Fig. 3f. The cardiomyocytes of the model were not long spindle with regular arrangement, but appeared irregular interstitial spaces (as indicated by the arrows in Fig. 3f). The shape of cardiomyocytes had been improved to long spindle in good order with no meaningless intercellular cavities when treated with CAPE or CAPE-\(p\)NO\(_2\).

3.4 Effects on expression of TGF-B1 and Gal-3 in the CMI rats

The results of immunohistochemistry showed that the expression of TGF-\(\beta\)1 in CMI rats enhanced significantly \((p < 0.01)\), and the level of TGF-\(\beta\)1 decreased after CAPE or CAPE-\(p\)NO\(_2\) treatment \((p < 0.01)\) (Fig. 4a). From Western Blot results, it was visible that the level of TGF-\(\beta\)1 in heart was up-regulated in the model rats \((p < 0.01)\), and could be down-regulated after treatment of CAPE or CAPE-\(p\)NO\(_2\) \((p < 0.01)\) (Fig. 4d). The decreasing effect of CAPE was better than that of High-\(p\)NO\(_2\) in both immunohistochemistry and western blot results \((p < 0.05)\).

As in Fig. 4b, the level of Gal-3 in CMI rat heart increased significantly \((p < 0.01)\), and decreased after CAPE or CAPE-\(p\)NO\(_2\) treatment \((p < 0.01)\). The effect of High-\(p\)NO\(_2\) was better than CAPE \((p < 0.01)\). The Gal-3 levels of serum and heart exhibited similar trend to immunohistochemistry result both before and
after CAPE or CAPE-\(\text{pNO}_2\) treatment, while the content of Gal-3 in Low-\(\text{pNO}_2\) group was approaching or lower than that in CAPE group in the results of serum ELISA assay and western blot test (Fig. 4c and d).

3.5 CAPE-\(\text{pNO}_2\) attenuated cardiac fibrosis in the CMI rats

The results of Sirius red staining indicated that the level of collagen deposition in the heart of CMI rats was higher than that of the control (\(p < 0.01\)). After treatment of CAPE or CAPE-\(\text{pNO}_2\), the collagen deposition was significantly reduced (\(p < 0.01\)). The High-\(\text{pNO}_2\) displayed better effect than CAPE (\(p < 0.05\)) (Fig. 5a).

The expression of MMP-9, Smad2, Col-I and Col-III was increased in the heart of model rats (\(p < 0.01\)). After treatment with CAPE or CAPE-\(\text{pNO}_2\), the expression of these proteins decreased (\(p < 0.05\)), and the effect of High-\(\text{pNO}_2\) was more potent than CAPE (\(p < 0.05\)). The effects of Low-\(\text{pNO}_2\) on MMP-9 and Smad2 were stronger compared with CAPE (\(p < 0.05\)), but weaker on Col-I and Col-III (\(p < 0.01\)) (Fig. 5b).

3.6 CAPE-\(\text{pNO}_2\) decreased cardiomyocytes apoptosis in the CMI rats

Results of TUNEL staining manifested that apoptosis of cardiomyocytes in CMI rat increased (\(p < 0.01\)); CAPE and CAPE-\(\text{pNO}_2\) could effectively reduce the apoptotic rate of cardiomyocytes (\(p < 0.05\)), and the effect of High-\(\text{pNO}_2\) was similar to that of CAPE (Fig. 6a).

There was a rising trend of expressions of Bax and caspase 3, and a declining trend of Bcl-2 expression in the heart of CMI rat (\(p < 0.01\)). After treatment with CAPE or CAPE-\(\text{pNO}_2\), the expression of Bax and caspase 3 decreased (\(p < 0.01\)), and Bcl-2 expression increased. High-\(\text{pNO}_2\) showed better effect on these proteins than CAPE. The effects on cardiomyocytes apoptosis were in accordance with the TUNEL results (\(p < 0.05\)) (Fig. 6b).

3.7 CAPE-\(\text{pNO}_2\) weakened myocardial inflammation in the CMI rats

It was revealed in the immunohistochemistry results in heart that the expression of IL-6 was higher in the CMI rats than the control (\(p < 0.01\)), and it was reduced with the treatment of CAPE or CAPE-\(\text{pNO}_2\) (\(p < 0.01\)). High-\(\text{pNO}_2\) showed better effects than CAPE did (\(p < 0.05\)) (Fig. 7a).

As shown in Fig. 7b, CMI rats had higher expressions of TNF-\(\alpha\), NF-\(\kappa\)B, IL-1\(\beta\), IL-6 and PI3K in heart (\(p < 0.01\)). The expressions of these proteins can be down-regulated by CAPE and CAPE-\(\text{pNO}_2\), and the regulating effect of High-\(\text{pNO}_2\) was similar to or stronger than that of CAPE.

3.8 CAPE-\(\text{pNO}_2\) alleviated fibrosis, apoptosis and inflammation of lung in the CMI rats
As demonstrated in Fig. 8a, the TGF-β1 and Gal-3 expressions in CMI rats were significantly increased ($p < 0.01$), and it could be down-regulated by CAPE and CAPE-pNO$_2$ ($p < 0.05$). The effect of High-pNO$_2$ on Gal-3 was better than CAPE ($p < 0.05$). For MMP-9, Smad2, Col-I, Col-III, Bax, caspase 3, TNF-α, NF-κB and IL-6, there was an increasing trend of expressions in CMI rat ($p < 0.01$), and the expressions decreased with the treatment of CAPE or CAPE-pNO$_2$ ($p < 0.05$). In contrast, the level of Bcl-2 reduced in CMI rat ($p < 0.01$), and rose after CAPE or CAPE-pNO$_2$ treatment ($p < 0.05$). High-pNO$_2$ usually showed better results than CAPE (Fig. 8b, c and d).

### 3.9 CAPE-pNO$_2$ decreased TGF-B1 and Gal-3 expression in LPS-induced H9c2

As shown in Fig. 9, the results of immunofluorescence and western blot experiment indicated that the content of TGF-β1 and Gal-3 in model cells stimulated by LPS were increased significantly ($p < 0.01$), and the level of TGF-β1 and Gal-3 could be down-regulated after CAPE or CAPE-pNO$_2$ treatment. CAPE-pNO$_2$ showed better effects on Gal-3 than CAPE ($p < 0.01$). After co-treatment with CAPE-pNO$_2$ and MCP, the decreasing effect on Gal-3 was not as good as treatment of CAPE-pNO$_2$ alone ($p < 0.01$). While the effect of CAPE-pNO$_2$ on TGF-β1 was approaching to CAPE or co-treatment with CAPE-pNO$_2$ and MCP in immunofluorescence and western blot experiment.

### 3.10 CAPE-pNO$_2$ attenuated fibrosis in LPS-induced H9c2

The results of immunofluorescence showed that Col-I and Col-III levels were markedly increased in LPS-induced H9c2 ($p < 0.01$). CAPE and CAPE-pNO$_2$ treatment decreased Col-I and Col-III expression, and CAPE-pNO$_2$ showed the similar effect on Col-III ($p > 0.05$) and showed the better effect on Col-I ($p < 0.01$) than CAPE. While the down-regulation effect of CAPE-pNO$_2$ decreased after MCP treatment (Fig. 10a and b). Western blot results of Col-I and Col-III showed the similar regulation trend as immunofluorescence (Fig. 10c).

### 3.11 CAPE-pNO$_2$ attenuated apoptosis in LPS-induced H9c2

The results of AO staining exhibited that the number of surviving cells decreased and apoptosis appeared in LPS-induced H9c2. CAPE-pNO$_2$ could increase the viable cell quantity and reduce the LPS-induced apoptosis. However, after treatment with MCP suppressed the protection effect of CAPE-pNO$_2$ (Fig. 11a).

Western blot assay analysis showed the Bax and caspase 3 up-regulations, and Bcl-2 down-regulations in LPS-induced H9c2 ($p < 0.01$). CAPE and CAPE-pNO$_2$ treatment decreased the Bax and caspase 3 expressions, and increased Bcl-2 expression. Co-treatment with MCP and CAPE-pNO$_2$ eliminated the effect of CAPE-pNO$_2$ ($p < 0.01$, Fig. 11b).

### 3.12 CAPE-pNO$_2$ weakened inflammation in LPS-induced H9c2
In immunofluorescence and western blot results, TNF-α and IL-6 levels were significantly increased after the LPS injury \((p < 0.01)\). TNF-α and IL-6 were markedly down-regulated after CAPE or CAPE-\(p\)NO\(_2\) treatment \((p < 0.01)\). However, the expression of TNF-α and IL-6 in MCP-\(p\)NO\(_2\) group were significantly higher than that in CAPE-\(p\)NO\(_2\) group \((p < 0.05)\). In addition, CAPE-\(p\)NO\(_2\) had a better effect on down regulating TNF-α than CAPE \((p < 0.05, \text{Fig. 12})\).

4 Discussion

Myocardial ischemia is one of the main causes of cardiac remodeling and heart failure [31], which is also closely related to atherosclerosis and myocardial infarction. The preconditioning and treatment on chronic and acute ischemia have clinical significance in the treatment of coronary artery disease [32]. Many cardiovascular pathological changes can lead to MI, including oxidative stress, inflammation, fibrosis and apoptosis, and these changes are also related to variety of activating proteins and factors, such as NF-κB, TNF-α and TGF-β1 [33].

CAPE-\(p\)NO\(_2\) was synthesized based on CAPE in our lab, and it showed better protective effects on heart of rats with MIRI [21, 23]. Therefore, in this study, CAPE was considered as a positive control, and the doses of CAPE-\(p\)NO\(_2\) were set to be consistent with or lower than that of CAPE.

Our results demonstrated that CAPE-\(p\)NO\(_2\) could decrease the lung index, the contents of TC, TG and LDL in the serum of CMI rat, as well as increase the body weight, heart index and the level of HDL. This compound could better promote lipid metabolism and growth, and improve the lipid accumulation in the heart of the model rats. Besides, CAPE-\(p\)NO\(_2\) also reduced the serum levels of cardiac enzyme including HBDB, LDH, CK and CK-MB in CMI rats, which were positively correlated with cardiac damage. CAPE-\(p\)NO\(_2\) treatment improved the disordered arrangement of cardiomyocytes and irregular cavities in HE staining figures, which means the cardiac structure has changed to recovery.

Studies have confirmed that high heart rate can aggravate myocardial ischemia, ventricular arrhythmia, vascular oxidative stress, endothelial dysfunction and atherosclerosis [34], and lowering the heart rate can improve regional blood flow and systolic function of ischemic myocardium [35]. ECG is usually used in clinical diagnosis and monitoring of cardiovascular diseases such as myocardial ischemia and myocardial infarction [36], of which elevated ST segment of ECG is a sensitive and specific marker [37]. QRS complex waves also produce electrical changes during depolarization of ischemic ventricular tissue [38]. After treatment with CAPE-\(p\)NO\(_2\), the heart rate of CMI rats became slower, elevated ST segment dropped, and the positive and negative charge of the QRS wave group tended to be balanced. Thus CAPE-\(p\)NO\(_2\) treatment exhibited repair efficacy on heart function.

TGF-β1 is closely related to heart disease, which aggravates cardiomyocyte apoptosis and cardiac hypertrophy, and it also takes part in myocardial fibrosis [39]. TGF-β1 is up-regulated in remodeling after myocardial infarction and heart failure, and it can intensify MIRI [5]. In this study, the level of TGF-β1 increased in CMI heart and LPS-induced H9c2, and CAPE-\(p\)NO\(_2\) could significantly decrease the
expression of TGF-β1. Therefore, it is probable that the inhibition effect of CAPE-pNO2 on cardiomyocyte apoptosis and myocardial fibrosis would be related to the decreased expression of TGF-β1 in heart tissue.

Gal-3 has important significance in the diagnosis, prognosis and treatment of cardiovascular diseases in clinic. Higher serum and plasma Gal-3 levels are related to more serious MI and fibrotic cardiomyopathy [40, 41]. In this study, the contents of Gal-3 increased significantly in CMI rat heart tissue, serum and LPS-induced H9c2, and CAPE-pNO2 significantly decreased Gal-3 levels.

To further explore the mechanism of cardio-protective effect of CAPE-pNO2, we co-treated with Gal-3 inhibitor MCP and CAPE-pNO2. Not as anticipation, the CAPE-pNO2-mediated Gal-3 decrease was eliminated. We speculate that co-treated with MCP and CAPE-pNO2 may have competitive binding with Gal-3, or inhibit the effect of CAPE-pNO2 in other ways, which needs further studied.

Gal-3 participates in fibrosis and it can increase the accumulation of Col-Ia in the fibrosis of pulmonary adventitia fibroblasts induced by TGF-β1 [42]. Besides, Gal-3 is the binding substrate of MMP-9. In our study, the collagen accumulation in the heart of CMI rats was significantly decreased after CAPE-pNO2 treatment, along with a decrease of MMP-9, Smad2, Col-I and Col-III. Similarly, this effect was inhibited by MCP in LPS-induced H9c2. It has been reported that TGF-β1 regulates the expression of Gal-3 in nuclear pulposus cells through a typical Smad3 signaling pathway [43]. Moreover, in mice deficient in galectin-3, TGF-β1 and bleomycin-induced lung fibrosis was dramatically reduced [44], which also confirmed that Gal-3 mediated the actions of TGF-β1, and that Gal-3 was the downstream protein of TGF-β1. Thus we call it TGF-β1/Gal-3 pathway in this article for short. It can be inferred from the above experimental results and literature that the effect of CAPE-pNO2 on alleviating myocardial fibrosis in CMI was by regulating MMP-9 and TGF-β1/Gal-3/Col pathway.

Overexpression of Gal-3 has been reported to reduce myocardial cell viability and induce apoptosis [45]. The expression of proliferating cell nuclear antigen and Bcl-2 decreased in cardiomyocytes with high expression of Gal-3, while the expression of Bax and caspase 3 increased [15]. In the present study, CAPE-pNO2 treatment significantly reduced the apoptosis rate of cardiomyocytes, up-regulated the expression of Bcl-2, and down-regulated the expression of Bax and caspase-3 in vivo and in vitro, while its effect was inhibited by MCP in LPS-induced H9c2. It implied that the anti-apoptosis effect of CAPE-pNO2 on the heart of CMI rats might be through the TGF-β1/Gal-3/Bcl-2 signaling pathway.

Myocardial ischemic injury, such as MIRI, is closely related to inflammatory response [46]. Furthermore, Cardiac inflammation increases the level of Gal-3 in the heart and releases it into the systemic circulation [41]. Gal-3 regulates typical inflammatory factors like TNF-α and IL-6 [47]. In this study, CAPE-pNO2 decreased the level of IL-6 expression in CMI heart, and significantly decreased the contents of some typical inflammatory related factors like TNF-α, NF-κB, IL-1β, IL-6 and PI3K. In LPS-induced H9c2, CAPE-pNO2 ameliorated inflammatory by suppressing the expression of IL-6 and TNF-α, but this effect was eliminated after treating with MCP. It can be concluded that CAPE-pNO2 effectively weaken myocardial
inflammation in CMI rats and LPS-induced H9c2, and the effect might be through TGF-β1/Gal-3/TNF-α and TGF-β1/Gal-3/IL-6 pathways.

Because of the circulation, the pathological changes of lung and heart are always correlated. For example, after myocardial ischemia and reperfusion, pulmonary congestion may be caused by delayed production of reactive nitrogen species, and acute lung injury in diabetic rats can be caused after myocardial ischemia-reperfusion [48, 49]. Moreover, in our study, increase of lung index in CMI rats could be seen, and it may be due to inflammation and other injury to enlarge lung. Further study found that the level of Gal-3, TGF-β1, MMP-9, Smad2, Col-I, Col-III, Bax, caspase 3, TNF-α, NF-κB and IL-6 were increased, and the level of Bcl-2 was decreased in CMI rats. CAPE-pNO₂ could up-regulate the expression of Bcl-2 and down-regulate the other proteins. The changing trend of these proteins was consistent with that in the heart. It came to a conclusion that CMI also induced the lung injury, and CAPE-pNO₂ could down-regulate TGF-β1 and Gal-3 in the lung of CMI rat, too. In addition, CAPE-pNO₂ could alleviate pulmonary fibrosis, apoptosis and inflammation through TGF-β1/Gal-3 pathway.

In conclusion (Fig. 13), CAPE-pNO₂ could significantly reduce the expression of Gal-3 and TGF-β1 in the heart, lung of CMI rats and LPS-induced H9c2, and had regulatory effect on related downstream proteins of Gal-3. These results showed that the protective effect of CAPE-pNO₂ on CMI may be via TGF-β1/Gal-3 pathway.

Declarations

Conflict of interest

The authors declare that there is no conflict of interest.

Ethics approval

All the animal protocols were consistent with the National Institutes of Health (NIH) guidelines and approved by the Ethical Committee for Animals of Southwest University.

Author contributions

Li Z, Zhang L and Li B designed the project; Zhang L, Wan Q and Han Y performed the experiments; Zhang L, Li Z, Li B and Wan Q wrote the manuscripts; Zhang L analyzed and interpreted data; all authors reviewed the manuscript.

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Code availability
Not applicable.

Consent to participate

Not applicable.

Data availability

Data supporting the results of this research are available to the corresponding author on request.

Consent for publication

Not applicable.

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**Figures**

**Figure 1**

CAPE-pNO2 increased body weight, heart index and decreased lung index in CMI rats. (a) The changes of body weight during 13 weeks. (b) The changes of heart index. (c) The changes of lung index. Data are
expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; ##p < 0.01 vs. model group; &&p < 0.01 vs. CAPE group; $$p < 0.01 vs. High-pNO2 group.

Figure 2

CAPE-pNO2 decreased serum lipid indexes including TC, TG and LDL level, and increased the level of HDL. (a) Serum TC level. (b) Serum TG level. (c) Serum HDL level. (d) Serum LDL level. Data are expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &p < 0.05 vs. CAPE group; $p < 0.05, $$p < 0.01 vs. High-pNO2 group.
Figure 3

CAPE-pNO2 promoted the cardiac function recovery and structure improvement in CMI rats. (a) Changes of electrocardiogram. (b-e) Changes of serum level of cardiac injury-related enzymes, including HBDB, LDH, CK and CK-MB. (f) The paraffin section of heart tissue stained with haematoxylin and eosin. The cells in the control group were arranged regularly and in a long spindle shape, but the cells in the model group were disordered, with irregular interstitial spaces appeared (as indicated by the arrows in Fig. 3f). Scare bar = 100 µm. Data are expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &p < 0.05 vs. CAPE group.
Figure 4

CAPE-pNO2 down-regulated TGF-β1 level in heart, as well as the expression of Gal-3 in heart, serum. (a-b) The paraffin section of heart tissue detected by immunohistochemistry to measure TGF-β1 and Gal-3 expression. The sections were incubated with primary antibody (anti-TGF-β1 and anti-Gal-3) and goat anti-rabbit IgG antibody conjugated with HRP, then re-dyed with DAB and hematoxylin. The density value of brown part was used to measure the expression level of TGF-β1 and Gal-3. Scare bar = 100 µm. (c) Serum Gal-3 level. (d) The expressions of TGF-β1 and Gal-3 in the heart of CMI rats detected by western blot. Data are expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &p < 0.01, &&p < 0.01 vs. CAPE group; $$p < 0.01 vs. High-pNO2 group.
CAPE-pNO₂ attenuated cardiac fibrosis in CMI rats. (a) Levels of collagen deposition of the paraffin section of heart tissue stained with Sirius red. The density value of red part was used to measure the collagen deposition. Scare bar = 100 µm. (b) The expressions of MMP-9, Smad2, Col-I and Col-III in the heart of CMI rats detected by western blot. Data are expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &p < 0.05, &&p < 0.01 vs. CAPE group; $p < 0.05, $$p < 0.01 vs. High-pNO₂ group.
CAPE-pNO2 decreased cardiomyocytes apoptosis in CMI rats. (a) The paraffin section of heart tissue detected by TUNEL staining. TUNEL positive cell rate was used to measure cardiomyocyte apoptosis. It was calculated by the ratio of apoptotic cell number to total cell number (the apoptotic cell nucleus was reddish brown, the normal cell nucleus was blue). Scale bar = 100 µm. (b) The expressions of Bcl-2, Bax, and caspase 3 in the heart of CMI rats detected by western blot. Data are expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &&p < 0.01 vs. CAPE group; $$p < 0.01 vs. High-pNO2 group.
CAPE-pNO2 weakened cardiac inflammation in CMI rats. (a) The paraffin section of heart tissue detected by immunohistochemistry to measure IL-6 expression. The sections were incubated with primary antibody (anti-IL-6) and goat anti-rabbit IgG antibody conjugated with HRP, then re-dyed with DAB and hematoxylin. The density value of brown part was used to measure the expression level of IL-6. Scare bar = 100 µm. (b) The expressions of TNF-α, NF-κB, IL-1β, IL-6 and PI3K in the heart of CMI rats detected by western blot. Data are expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; ##p < 0.01 vs. model group; &p < 0.05, &&p < 0.01 vs. CAPE group; $p < 0.05, $$p < 0.01 vs. High-pNO2 group.
Figure 8

CAPE-pNO2 alleviated fibrosis, apoptosis and inflammation of lung. (a) The expressions of TGF-β1 and Gal-3 in lungs detected by western blot. (b) The expressions of fibrosis related proteins (MMP-9, Smad2, Col-I and Col-III) in the lung of CMI rats detected by western blot. (c) The expressions of apoptosis related proteins (Bcl-2, Bax and caspase 3) in the lung of CMI rats detected by western blot. (d) The expressions of inflammation related proteins (TNF-α, NF-κB and IL-6) in the lung of CMI rats detected by western blot. Data are expressed as mean ± SD and repeated three times (n = 6). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &p < 0.05, &&p < 0.01 vs. CAPE group; $p < 0.05, $$p < 0.01 vs. High-pNO2 group.
Figure 9

CAPE-pNO2 down-regulated TGF-β1 and Gal-3 expression in LPS-induced H9c2. (a-b) The expressions of TGF-β1 and Gal-3 in cells detected by immunofluorescence. Scale bar = 100 µm. (c) Expression of TGF-β1 and Gal-3 in cells detected by western blot. Data are expressed as mean ± SD (n=3). **p < 0.01 vs. control group; ##p < 0.01 vs. model group; &&p < 0.01 vs. CAPE group; $$p < 0.01 vs. CAPE-pNO2 group.
CAPE-pNO2 attenuated fibrosis in LPS-induced H9c2. (a-b) The expression of Col-I and Col-III in cells detected by immunofluorescence. Scale bar = 100 μm. (c) Expression of Col-I and Col-III in cells detected by western blot. Data are expressed as mean ± SD (n=3). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &p < 0.05, &&p < 0.01 vs. CAPE group; $$p < 0.01 vs. CAPE-pNO2 group.

Figure 10
CAPE-pNO2 decreased apoptosis in LPS-induced H9c2. (a) AO staining results. Scale bar = 100 µm. (b) Expression of Bcl-2, Bax and caspase 3 in cells detected by western blot. Data are expressed as mean ± SD (n=3). **p < 0.01 vs. control group; #p < 0.05, ##p < 0.01 vs. model group; &p < 0.05, &&p < 0.01 vs. CAPE group; $$p < 0.01 vs. CAPE-pNO2 group.
CAPE-pNO2 weakened inflammation in LPS-induced H9c2. (a-b) The expression of TNF-α and IL-6 in cells detected by immunofluorescence. Scale bar = 100 µm. (c) Expression of TNF-α and IL-6 in cells detected by western blot. Data are expressed as mean ± SD (n=3). **p < 0.01 vs. control group; ##p < 0.01 vs. model group; &p < 0.05 vs. CAPE group; $p < 0.05, $$p < 0.01 vs. CAPE-pNO2 group.
Figure 13

Experimental design. The experiment aimed to explore the effect of CAPE-pNO2 on the expression of Gal-3 and on CMI rat. It's reported that TGF-β1 can regulate the Gal-3 expression, so we regarded TGF-β1/Gal-3 as an existing pathway, and it can regulate many related downstream proteins. Considering the pathway and the pathological mechanism of CMI, this article mainly discussed the protective effects of CAPE-pNO2 on CMI rats in terms of fibrosis, apoptosis and inflammation. The red lines represented the inhibiting effects, and the green arrows represented the promoting effects.