Mechanism of H_2S-mediated ROCK inhibition of total flavones of *Rhododendra* against myocardial ischemia injury

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Abstract. Our previous studies have indicated that pretreatment with total flavones of *Rhododendra* flower (TFR) may protect against myocardial ischemic injuries in rats and mice. The cystathionine γ-lyase/hydrogen sulfide (CSE/H_2S) pathway have been associated with several cardiovascular diseases, but the effect of TFR on the Rho-associated protein kinase (ROCK) and CSE/H_2S signaling pathways remains unknown. In the present study, the protective effects of TFR as a ROCK inhibitor in a mice model of myocardial infarction induced by isoproterenol (ISO) were investigated, and the hearts from the wild type and CSE knockout (KO) mice were examined. It was identified that the CSE KO mice exhibited decreased levels of ST segment elevation following anoxia/reoxygenation damage, increased LDH and CK-MB levels, aggravated pathological damage, and increased ROCK1, ROCK2 and MLC1 protein levels. In the CSE KO mice, there were no marked changes of the above experimental results between the TFR group and the model group. These results suggested that TFR-based inhibition of the RhoA/ROCK signal pathway may be mediated by the CSE-H_2S signalling pathway and may be a novel therapeutic target for myocardial ischemia injury.

Introduction

Hydrogen sulfide (H_2S) is a highly dispersive gasotransmitter that affects cells and organs function through different mechanisms (1). H_2S is increasingly being considered as an important signaling molecule in the cardiovascular systems (2,3). Endogenous production of H_2S is primarily catalyzed by cystathionine β-synthase, cystathionine-γ-lyase (CSE) and 3-mercaptosulphurtransferase (4). Among them, CSE is the primary H_2S-producing enzyme in cardiovascular tissues. The disordered metabolism and functions of the CSE/H_2S pathway have been associated with several cardiovascular diseases, including A/R injury, hypertension, atherosclerosis and oxidative stress (5-9).

Rho-associated protein kinase (ROCK), the best-characterized effector of the small G protein Rho, has been proposed to be potential targets in the therapy of cardiovascular diseases (10,11). Various studies have indicated that ROCK inhibitors prevent the progress of myocardial infarction by hemodilution, vascular dilation and inhibition of neutrophil accumulation (11-13). The useful effects of ROCK inhibition against A/R damage using the ROCK inhibitors fasudil and Y-27632 have been established (14,15). This suggests that ROCK serves a vital role in myocardial infarction.

Total flavones of *Rhododendra* flower (TFR), an effective compound extracted from the *Rhododendra* flower, is comprised of flavones including quercetin, hyperin, rutin and other flavonoids (16,17). Our previous studies have indicated that TFR has significant protective effects against myocardial ischemic injuries in rat and mice models (18,19), and that the protective mechanism may be engaged with the inhibition of ROCK1 and ROCK2 and activation of the potassium channel (20). Certain previous studies have suggested that flavonoid compounds may prevent the RhoA/ROCK signal pathway by decreasing the contractility of vascular smooth muscle cells (21-23).

In light of these data, the present study aimed to evaluate the cardiovascular protective effects of TFR as a ROCK inhibitor in a mice model of myocardial infarction induced by isoproterenol. The hearts from wild-type (WT) and CSE knockout (KO) mice were examined. During the process of myocardial ischemia-reperfusion injury, the effect of endogenous H_2S on ROCK signaling pathways was explored, and the effect of TFR on the ROCK and CSE/H_2S signaling pathways was investigated.

Materials and methods

Drugs and reagents. TFR (content of flavones >85%) was provided by Hefei Heyuan Medical Company Technology Co., Ltd. Isoprenaline (ISO) was produced by Shanghai Hefeng

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Abbreviations: H_2S, hydrogen sulfide; ROCK, Rho-associated protein kinase; A/R, anoxia/reoxygenation; TFR, Total flavones of *Rhododendra* flower

Key words: total flavones of *Rhododendra* flower, hydrogen sulfide, Rho-associated protein kinase, anoxia/reoxygenation

Pharmaceutical Co. Ltd. Lactate dehydrogenase (LDH, cat. no. A020-1-2) and creatinine kinase isoenzyme (CK-MB; cat. no. H197) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. Rabbit polyclonal primary antibodies against ROCK1 and ROCK2 were provided by EnoGene Biotech Co., Ltd. Membrane protein MLC1 (MLC1) was purchased from Santa Cruz Biotechnology, Inc.

Animal model. The present study was approved by the Ethics Committee for Animal Experiments of Anhui Medical University (no. 20160315). The CSE KO and wild-type mice (C57 strain) were produced by Shanghai Model Organisms Center. Wild-type and CSE KO mice (n=60; age, 10-16 weeks; weight, 18-24 g; half male and female) were used for the experiment (Fig. 1).

The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication, 8th edition, 2011) (24). WT and CSE KO mice (6 per group) were divided to 5 groups. ISO (0.002 mg/kg) was injected in mice. Group I served as control, with equal dose of normal saline. Myocardial infarction was induced in groups II-V by subcutaneous administration of ISO on the first, second and third days respectively. Group III-V received TFR orally (30, 60 or 120 mg/kg, respectively) once a day 5 min prior to the ISO for 3 days; group II received the equal dose of normal saline. The mice were anaesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg). The mice were confirmed to be fully anaesthetized when the breathing rate decreased and breathing depth increased, and the righting reflex, and eyelid and tail-pinch reflexes were lost. At the end of protocol, mice were sacrificed by anesthesia using 2% isoflurane, which was then increased to 5%. Then, cardiac puncture was performed by a qualified technician. Following cardiac puncture, the mice were observed for respiratory and cardiac arrest, pupil dilation and disappearance of the pupillary light reflex. Following these observations, the mice were confirmed to be dead within 6-10 min.

PCR. Tail tissue was used. DNA polymerase was used (2x Premix Tag; Takara Bio; cat. no. RR902Q). RNA extraction buffer and supplier used was TRIzol Reagent from Thermo Fisher Scientific, Inc. (cat. no. 15596). The RT kit used was iScript gDNA Clear cDNA Synthesis kit from Bio-rad Laboratories, Inc. (cat. no. 1725035).

DNA polymerase used and supplier sequences of the forward and reverse primers: P1: CCTGGATATAAGCGC CAAAG, P2: AGGAACCAGGGCGTATCTCT, P3: CGA GAATTCCATTGCTCAGG. Reverse transcription protocol was as follows: 94˚C for 3 min, 94˚C for 30 sec, 57˚C for 30 sec, 72˚C for 40 sec, 72˚C for 10 min then 12˚C. The length of the wild product was 167 bp and the length of the mutant product was 309 bp.

Determination of ST-segment elevation. Electrocardiograms (ECGs) recorded ST-segment elevation at 5, 10, 15, 20 and 60 min following the final injection of ISO or normal saline. ECGs were recorded under 30 mg/kg pentobarbital sodium anesthetization administered by intraperitoneal injection. using needle electrodes and a Biological Function Experiment System (Chengdu Thaimeng Technology Co. Ltd). The recorded original data were estimated by the commercial software included in the acquisition system (AqDAnalysis 7; Lynx Tecnologia Ltda.).

Measurement of LDH and CK-MB levels. The supernatant was centrifuged at 3,000 x g for 10 min at 4˚C, the LDH and CK-MB levels were detected at 550 and 440 nm, respectively, by spectrophotometry according to the manufacturer's protocols of the assay kits. The experiment was repeated 3 times.

Histology. Left ventricular tissues were surgically removed, fixed in 10% buffered formalin at room temperature for 24 h, embedded in paraffin and sliced into 5-μm thick sections. The slides were stained with hematoxylin and eosin (H&E) for 5 min at room temperature and examined using a confocal microscope (magnification, x400; Olympus BX51; Olympus Corporation). The Rona classification standard (25) was used to evaluate the degree of myocardial tissue damage.

Western blot analysis. The left ventricular tissues from the mice were removed and placed in ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (50 μg) were separated on 10% polyacrylamide-Tris gels (Beyotime Institute of Biotechnology), transferred onto polyvinylidene difluoride membranes and blocked with 5% skim milk in TBST for 1 h at room temperature. Then, the membranes were incubated at 4˚C overnight with rabbit polyclonal antibodies against ROCK1 (1:1,000; cat. no. E1A7016), ROCK2 (1:1,000; cat. no. E1A6028) or MLC1 (1:1,000; cat. no. SC-86740) or monoclonal antibody against ß-actin (Bioworld Technology, Inc.). Following incubation with an anti-rabbit second antibody (OriGene Technologies, Inc.; 1:10,000 dilution in 5% skim milk) for 1 h at room temperature, the immunocomplexes were visualized using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.). The intensity of the immunoreactive bands were quantified by using the ImageJ analysis software (v.1.8.0; National Institutes of Health).

Measurement of RhoA activity. To detect the activity of RhoA, left ventricular tissues from the mice were lysed with radioimmunoprecipitation assay lysis buffer and incubated with 50 μg of the Rhotekin-RBD beads, containing a Rho-GTPase binding domain, at 4˚C for 1 h. The samples were then centrifuged at 5,000 x g at 4˚C for 1 min and the supernatant was removed. The beads were removed following washing with wash buffer. The remaining bead pellets were boiled with 200 μl 2X Laemmli sample buffer (Bio-Rad Laboratories, Inc.) at 85˚C for 5 min. Then, RhoA activity levels were determined using commercially available absorbance-based G-LISA RhoA activation assay kits (cat. no. BK 036-S; Cytoskeleton, Inc.). The left ventricular tissues were homogenized in lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentrated according to the manufacturer's protocol. following indirect immunodetection, RhoA activities were detected by measuring absorbance at 490 nm using a microplate spectrophotometer.

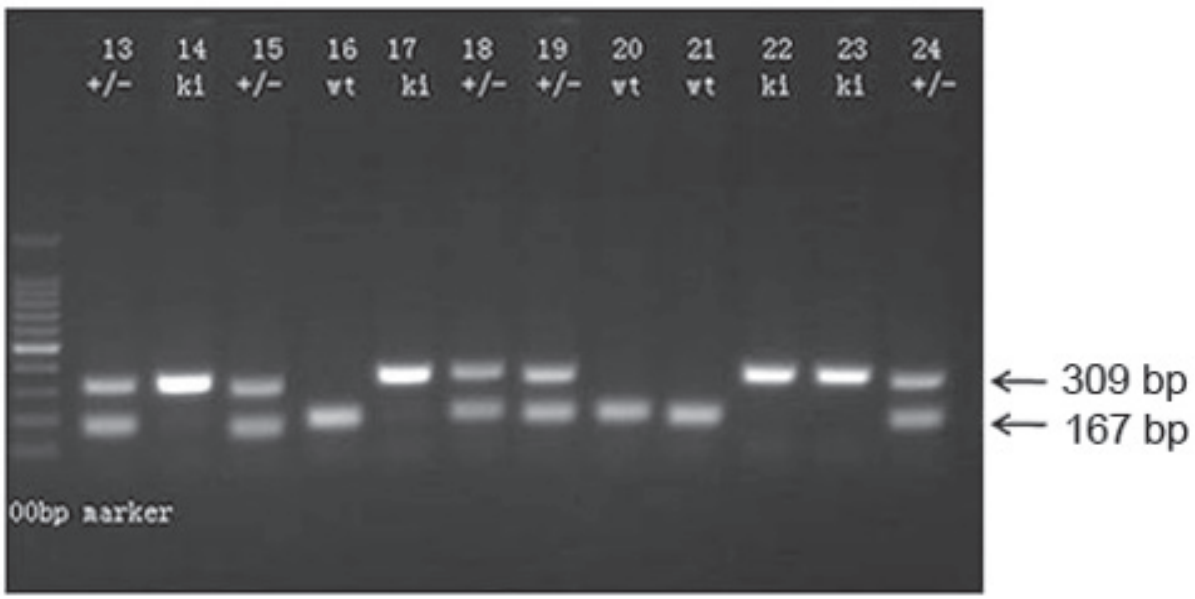

Figure 1. Polymerase chain reaction identification of CSE gene expression in mice. Lanes 14, 17, 22 and 23, CSE knockout mice (309 bp). Lanes 16, 20 and 21, wild type mice (167 bp). Lanes 13, 15, 18, 19 and 24, heterozygous mice (two bands). CSE, cystathionine γ-lyase.

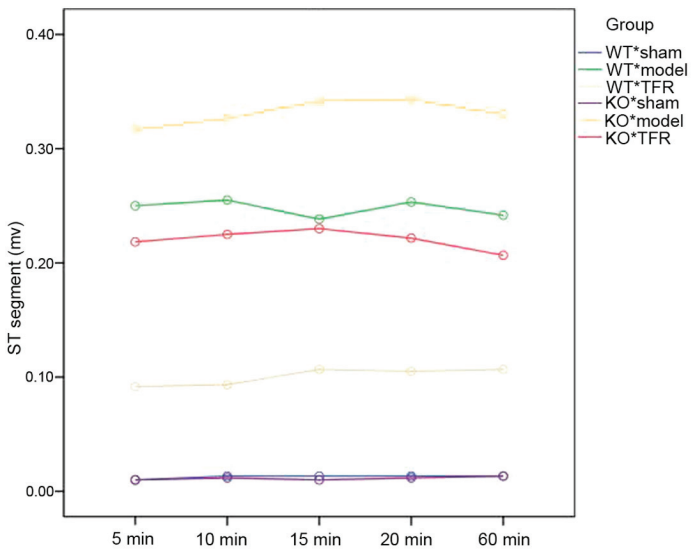

Figure 2. Effect of total flavones of *Rhododendra* flower on ST-segment elevation following ISO injection. Standard lead II of the ECG was recorded at 5, 10, 15, 20 and 60 min following the final injection of ISO. All values are presented the mean ± standard deviation. ISO, isoproterenol; WT, wild type; KO, knockout.

Statistical analysis. Data are expressed as means ± standard deviation, and differences between groups were analyzed by SPSS v15.0 (SPSS, Inc.). Statistical analyses were performed with one-way analysis of variance followed by the Duncan post-hoc test to determine the differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

The statistical analysis of pathology ranking data was performed using a Kruskal-Wallis H test. To determine the differences between 5 groups, the Bonferroni method was used. The difference between the WT and KO groups was analyzed using a Student's t-test for paired design analysis. $P<0.005$ was considered to indicate a statistically significant difference.

Results

Effect of TFR on ST-segment elevation. The ST segment of ECG in the model group of the wild type (WT) mice increased significantly at 5, 10, 15, 20 and 60 min after the final injection of ISO compared with the sham group ($P<0.01$; Figs. 2 and 3). Administration of 60 mg/kg TFR markedly decreased ST-segment elevation compared with the WT model group ($P<0.05$; Table I) and 120 mg/kg TFR markedly decreased ST-segment elevation compared with the WT model group ($P<0.01$; Table I).

The ST segment of ECG in the KO mice model group rose significantly at 5, 10, 15, 20 and 60 min following the final injection of ISO compared with the sham group ($P<0.01$;

Table I. Effect of TFR on the changes of ST segment (mV) of ECG in the CSE WT and KO mice.

A, WT group

	Time intervals, min				
Treatment groups	5	10	15	20	60
Control (n=6)	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
Model (n=6)	0.25±0.12[a]	0.25±0.14[a]	0.24±0.14[a]	0.25±0.15[a]	0.24±0.14[a]
TFR, mg/kg (n=6)					
30	0.19±0.14	0.19±0.12	0.19±0.14	0.20±0.14	0.21±0.13
60	0.12±0.11[b]	0.13±0.11[b]	0.13±0.12[b]	0.16±0.12[b]	0.16±0.13[b]
120	0.09±0.04[c]	0.10±0.05[c]	0.10±0.05[c]	0.11±0.06[c]	0.11±0.08[c]

B, KO group

	Time intervals, min				
Treatment groups	5	10	15	20	60
Control (n=6)	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
Model (n=6)	0.32±0.18[a]	0.33±0.19[a]	0.34±0.20[a]	0.34±0.18[a]	0.33±0.19[a]
TFR, mg/kg (n=6)					
30	0.28±0.19	0.30±0.19	0.27±0.18	0.28±0.20	0.27±0.18
60	0.26±0.17	0.26±0.16	0.27±0.18	0.26±0.15	0.26±0.15
120	0.22±0.10[d]	0.23±0.11[d]	0.23±0.10[d]	0.22±0.09[d]	0.21±0.10[d]

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. sham; [b]P<0.05 vs. model; [c]P<0.01 vs. model; [d]P<0.05 vs. WT TFR.

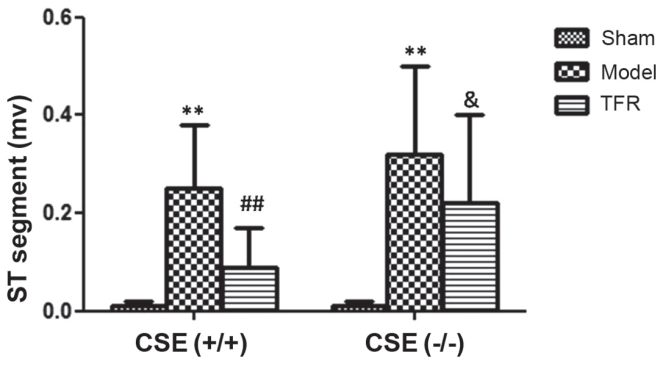

Figure 3. Effect of TFR on the changes of ST segment (mV) of ECG following subcutaneous isoproterenol injury in CSE WT and knockout mice. Data are presented as mean ± standard deviation (n=6). **P<0.01 vs. sham group. ##P<0.01 vs. model group. &P<0.05 vs. WT TFR group. TFR, total flavones of *Rhododendra* flower; CSE, cystathionine γ-lyase; WT, wild type; KO, knockout.

Figs. 2 and 3). However, no significant differences in the ST-segment elevation were observed following the administration of 30 and 60 mg/kg TFR compared with the model group in the KO mice (P>0.05), but the group of 120 mg/kg TFR was significant decreased compared with WT TFR group (P<0.05; Figs. 2 and 3; Table I).

In order to compare the elevations of the ST segments between the WT and KO mice more clearly, ST segment elevation was recorded at 5 min after the injection of ISO. The decrease in ST segment elevation in the TFR group in comparison with the model group was observed to be greater in the WT mice compared with the KO mice (P<0.05; Fig. 3).

Effect of TFR on the LDH and CK-MB level. Levels of LDH and CK-MB in the plasma supernatant are major indicators of myocardial anoxia/reoxygenation (A/R) injury. A few increases of LDH and CK-MB level were detected in A/R group of the WT mice (P<0.01). Treatment with 60 mg/kg TFR markedly inhibited the A/R-induced increases of LDH and CK-MB level in the plasma supernatant of the WT mice (P<0.01; Figs. 4 and 5).

Significant increases in LDH and CK-MB levels were detected in the A/R model group of the KO mice (P<0.01), and the LDH and CK-MB levels were increased significantly in A/R model group of the WT mice compared with the KO mice (P<0.01). Treatment with 60 mg/kg TFR had no effect of the A/R-induced increases in LDH and CK-MB level in the plasma supernatant of the KO mice (P>0.05). However, the LDH and CK-MB levels of the TFR group of the KO mice were significantly increased compared with those in the TFR group of the WT mice (Figs. 4 and 5).

Pathological observations. Analysis of the myocardium in the sham group in the WT mice population revealed a normal myofibrillar structure with stripes, branched appearance, and

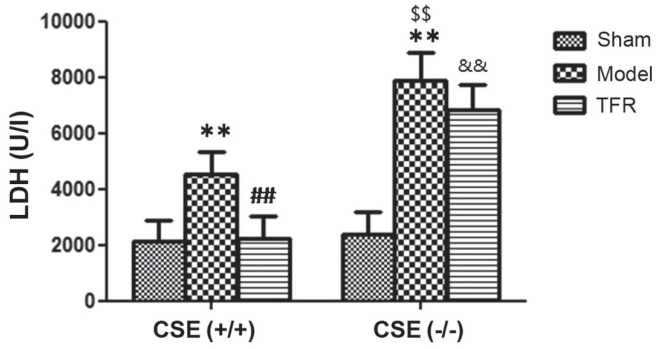

Figure 4. Effects of TFR treatment on increases in serum LDH levels in anoxia/reoxygenation-injured CSE KO mice. Data are presented as mean ± standard deviation (n=6). **P<0.01 vs. sham group. $^{\#\#}$P<0.01 vs. model group. $^{\$\$}$P<0.01 vs. WT model. $^{\&\&}$P<0.01 vs. WT TFR group. TFR, total flavones of *Rhododendra* flower; LDH, lactate dehydrogenase; CSE, cystathionine γ-lyase; WT, wild type; KO, knockout.

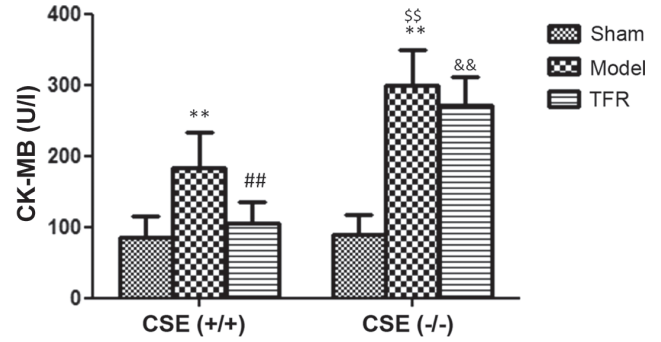

Figure 5. Effects of TFR on increases in serum CK-MB levels in anoxia/reoxygenation-injured CSE KO mice. Data are presented as mean ± standard deviation (n=6). **P<0.01 vs. sham group. $^{\#\#}$P<0.01 vs. model group. $^{\$\$}$P<0.01 vs. WT model. $^{\&\&}$P<0.01 vs. WT TFR group. TFR, total flavones of *Rhododendra* flower; CK-MB, creatinine kinase isoenzyme; CSE, cystathionine γ-lyase; WT, wild type; KO, knockout.

connections with adjacent myofibrils. In the mice treated with A/R, disorganized myocardium structure and loss of attachment between cardiomyocytes was observed. Tissues from the A/R mice exhibited obvious myocardial cell hypertrophy, cytopathy, loss of transverse striations and occasional cytoplasmic vacuolization. The TFR groups exhibited less severe histological damage, normal myocardial arrangement, clear transverse striations and fewer inflammatory cells.

The architecture of the myocardium was intact with erratic myofiber array in the sham group of the KO mice. Tissue from the A/R group of the KO mice revealed severely focal necrosis, myocardial cytopathy, loss of striations, severe infiltration of inflammatory cells and cytoplasmic vacuolization. Compared with this group, tissues from the A/R group of the WT mice population revealed less severe histological damage. In addition, the tissue sections from the TFR group of the KO mice demonstrated myocardial cell swelling, indistinct transverse striations and inflammatory cell infiltration. There were no marked differences in the pathological changes of 120 mg/kg TFR group of the KO mice compared with the A/R group of the same KO mice population (Fig. 6).

The pathological grades of myocardium from each group are presented in Table II. The level of significance was corrected as P-value of comparisons between different groups, when several comparisons were performed between groups. Analysis of the data demonstrated that there were significantly improvements in the pathological grades between the TFR and sham groups in the WT mice population (P<0.001). In addition, there were no significant changes between the TFR and sham groups in the KO mice population. Using a Z-test, it was demonstrated that there were significant improvements in the pathological changes of the 60 or 120 mg/kg TFR groups of the WT mice than the 60 or 120 mg/kg TFR groups of the KO mice (P<0.005). The results of Kruskal-Wallis H test in the WT and KO groups were χ^2=24.310 (P<0.001) and χ^2=21.858 (P<0.001), respectively (Table II).

Effect of TFR on ROCKs protein expression. The expression levels of ROCK1 and ROCK2 proteins were examined in each group (Fig. 7A), and were quantified by using densitometric analysis (Fig. 7B and C). Exposure to A/R markedly increased both ROCK1 and ROCK2 protein levels in the WT mice (P<0.01). The increases of ROCK1 and ROCK2 were markedly inhibited by treatment with 60 mg/kg TFR (P<0.01). Exposure to A/R significantly increased ROCK1 and ROCK2 protein levels in the KO mice (P<0.01). The increases of ROCK1 and ROCK2 were not markedly altered by treatment with 60 mg/kg TFR group of the KO mice compared with the A/R group of the KO mice. The results indicated that TFR treatment inhibited the expression of the ROCK proteins associated with the CSE/H_2S pathway.

The expression levels of MLC1 proteins were determined in each group (Fig. 8A), and levels of MLC1 proteins were quantified using densitometry (Fig. 8B). Exposure to A/R markedly increased MLC1 protein levels compared with the sham group in the WT mice (P<0.01). In addition, the increases of MLC1 were markedly inhibited by 60 mg/kg TFR compared with the A/R group of the WT mice (P<0.01). Exposure to A/R apparently increased MLC1 protein levels compared with the sham group of the KO mice (P<0.01). In the KO mice population, the inhibitory effect of TFR on the increased expression of MLC1 protein was significantly decreased compared with the TFR group of the WT mice population. These data demonstrated the TFR inhibited the expression of the MLC1 protein associated with the CSE/H_2S pathway.

Effect of TFR on RhoA activity. RhoA activity in the left ventricular tissues was detected using an absorbance-based G-LISA RhoA activation assay. As demonstrated in Fig. 9, RhoA activity in the model group (0.41±0.11) was significantly increased compared with that in the sham group (0.18±0.05) (P<0.01). In comparison with the model group, treatment with 60 mg/kg TFR markedly inhibited the increase in RhoA activity, which was decreased to 0.25±0.08 (P<0.01; Fig. 9).

Significant increases of RhoA activity were detected in the model group of KO mice (0.8±0.09) compared with the sham group (0.34±0.09) (P<0.01). In addition, the RhoA activity was increased significantly in the model group of KO mice compared with that in the WT mice (P<0.01). Compared with the model group, treatment with 60 mg/kg TFR had no effect on A/R-induced increases in RhoA activity of the KO mice (0.71±0.11) (P>0.05). However, in the KO mice, the RhoA activity of the TFR group was significantly increased compared with the TFR group of the WT mice (P<0.01; Fig. 9).

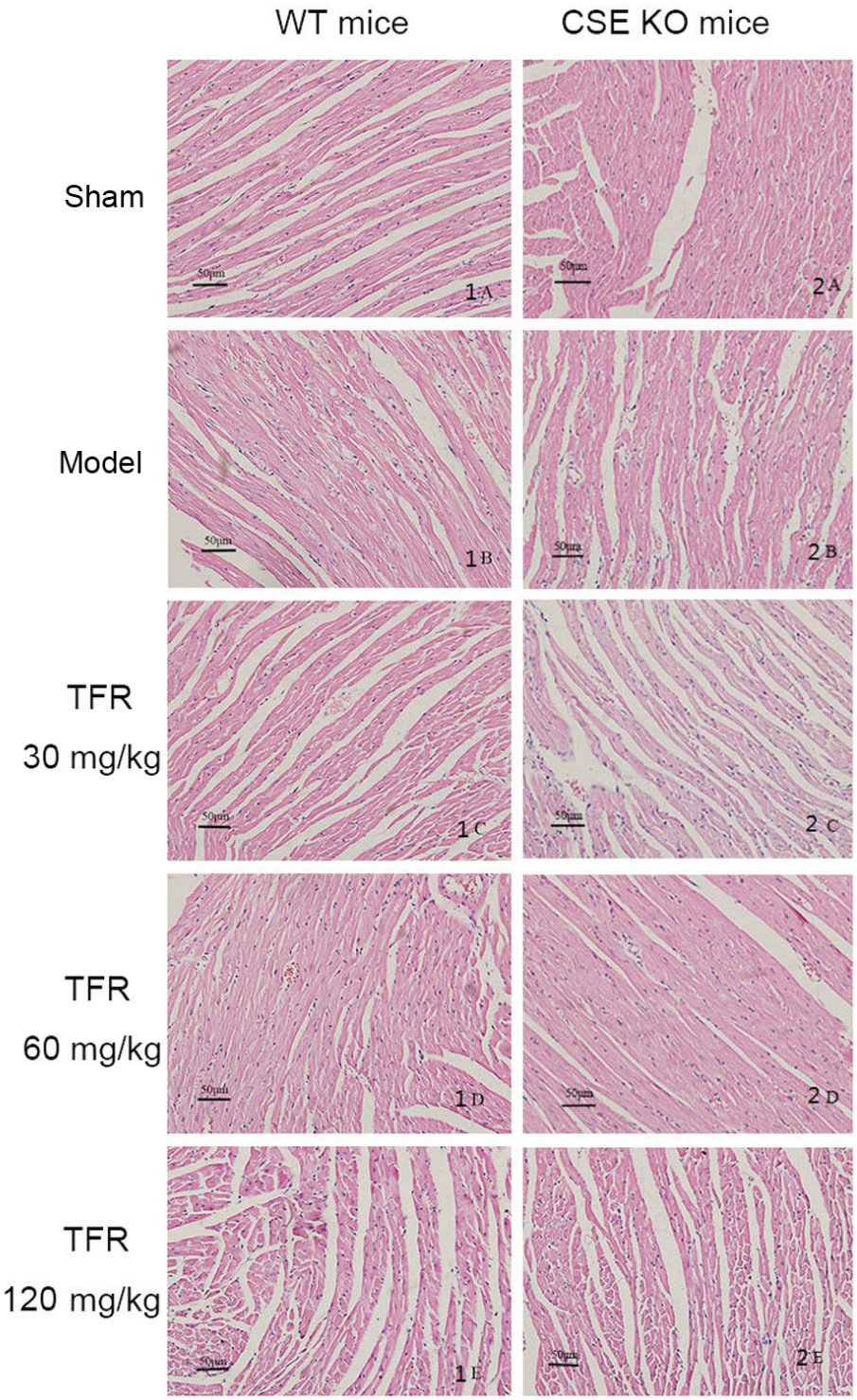

Figure 6. Histopathological observation of myocardium from WT and KO mice in an anoxia/reoxygenation model using hematoxylin and eosin staining. Magnification, x400. CSE, cystathionine γ-lyase; WT, wild type; KO, knockout; TFR, total flavones of *Rhododendra* flower.

Discussion

ISO is a synthetic β-adrenergic agonist that may cause serious stress in the cardiac muscle and necrosis of myocardium. Therefore, in the present study, ISO was used to induce acute myocardial ischemia. The acute myocardial ischemia caused by ISO was confirmed by loss of integrity of myocardial membranes on histological changes, increased ST segment elevation, and increased serum levels of LDH and CK-MB. In the present study, TFR treatment decreased the ST-segment elevation induced by ISO; TFR also decreased LDH and CK-MB levels in the serum. TFR treatment resulted in significant improvements in the pathological changes caused by hypoxia injury. The increases in expression levels of ROCK1, ROCK2 and MLC1 induced by the ISO were markedly inhibited by TFR treatment. These results suggested that TFR had cardioprotective effects in myocardial ischemia that may be attributed to the inhibition of the RhoA/ROCK signal pathway.

Table II. Pathological grades of cardiomyocytes in CSE WT and KO mice.

A, (WT group), CSE (+/+)

	Pathological grades, n					
Treatment group	0	I	II	III	IV	P-value
Sham	6	0	0	0	0	
Model	0	0	0	3	3	<0.005[a]
TFR, mg/kg						
30	0	0	2	3	1	<0.005[a]
60	0	3	2	1	0	<0.005[a],<0.005[b]
120	0	4	2	0	0	<0.005[a],<0.005[b]

B, (KO group), CSE (-/-)

	Pathological grades					
Treatment group	0	I	II	III	IV	P-value
Sham	6	0	0	0	0	
Model	0	0	0	2	4	<0.005[a]
TFR, mg/kg						
30	0	0	1	3	2	<0.005[a]
60	0	1	2	3	0	<0.005[a]
120	0	2	2	2	0	<0.005[a]

n=6. [a]P<0.005 vs. Sham group. [b]P<0.005 vs. Model group. Kruskal-Wallis H test showed that the difference of pathological grades among the 5 groups of WT mice were statistical significance (χ^2=24.310, P<0.001), the difference of Pathological grades among the 5 groups of KO mice were statistical significance (χ^2=21.858, P<0.001).

In the CSE KO mice, the ST-segment elevation induced by ISO was significantly increased compared with the WT mice. CSE KO mice also demonstrated increased LDH and CK-MB levels in the serum compared with the WT mice. CSE KO mice exhibited more severe pathological changes as a result of hypoxia injury compared with the WT mice, suggesting that H_2S was involved in the pathological process of myocardial ischemic injury. It was also observed that the expression levels of ROCK1, ROCK2 and MLC1 induced by ISO in the KO mice were markedly increased compared with the WT mice. These results suggested that CSE KO led to the decrease in H_2S expression and activation of the RhoA /ROCK signal pathway, which may have aggravated the myocardial ischemic injury. H_2S has protective effects against A/R injury in mice heart tissues by preventing the RhoA/ROCK signal pathway (20,26). TFR inhibition of the RhoA/ROCK signal pathway may be mediated by the CSE-H_2S pathway.

The study by Zhang *et al* (20) indicated that the cardioprotection afforded by TFR treatment involved the stimulation of nitric oxide release and the inhibition of lipid peroxidation. Increasing evidence has suggested that the RhoA/ROCK pathway serves an important role in the A/R damage, vascular smooth muscle cell proliferation, cardiac hypertrophy, heart failure and ventricular remodeling (27,28). Development of hypertension and myocardial infarction (MI); the two primary drivers of cardiovascular disease are associated with cardiac ROCK activation and phosphorylation of ROCK target proteins (29). ROCK inhibitors have a beneficial effect in attenuating hypertension and MI associated with ROCK activation (30). In addition, inhibition of the ROCK pathway may have a protective effect on cardiovascular function; the inhibitory agents Y27632 or fasudil were demonstrated to limit infarct size, alleviate the A/R damage, decrease the release of the MDA and LDH and promote the recovery of myocardial function following ischemia (31,32).

During agonist-induced vascular smooth muscle cell (VSMC) contraction, MLC phosphorylation is a crucial step for force development. ROCK, when activated by the small GTPase RhoA, inhibits MLC phosphatase (MLCP) activity by phosphorylating its myosin-binding subunit, thereby serving a key role in agonist-induced Ca2+ sensitization and VSMC hypercontraction (33).

The major regulatory mechanism of smooth muscle contraction is the phosphorylation/dephosphorylation of MLC (34). MLC is phosphorylated by the $Ca2^+$-calmodulin-activated MLC kinase (MLCK) and dephosphorylated by the $Ca2^+$-independent MLCP. Therefore, a rise in cytosolic $Ca2^+$ concentration produces smooth muscle contraction via the activation of MLCK and consequent phosphorylation of MLC (10). Hyperin is the primary active ingredient of TFR; it inhibits the contraction of the rabbit cardiac papillary muscle (35). In the present study, the increases of ROCK1, ROCK2 and MLC1

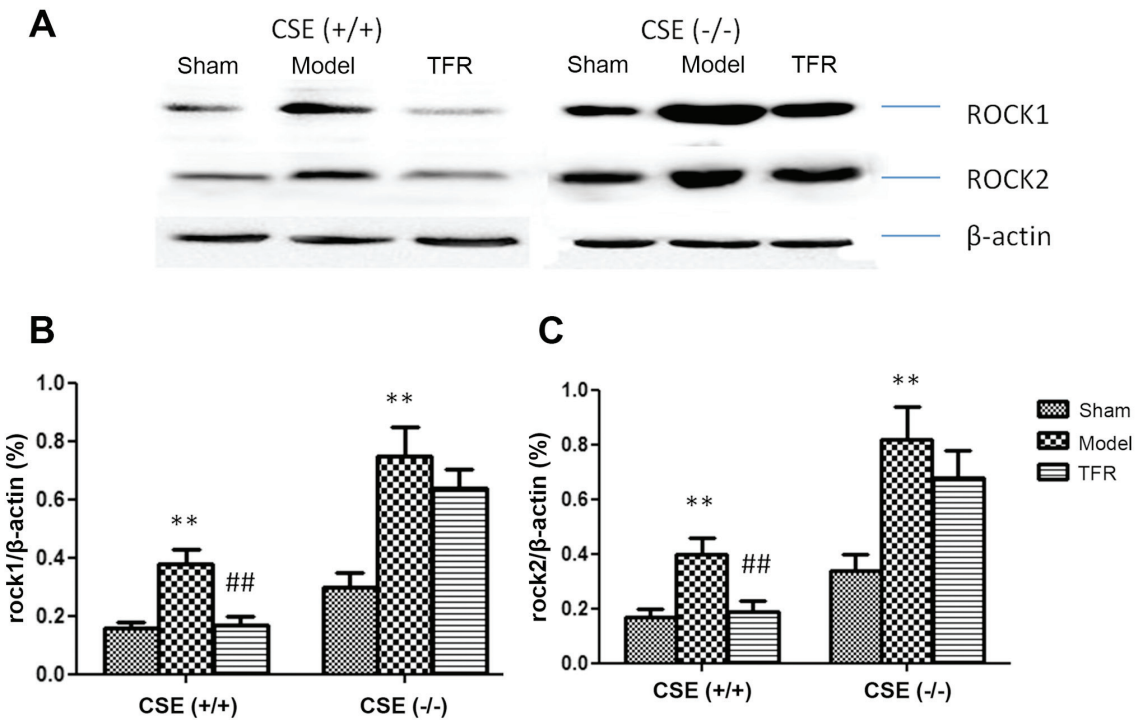

Figure 7. (A-C) Effect of TFR on the expression of ROCK1 and ROCK2 protein in isoproterenol-treated mice myocardium. **P<0.01 vs. sham group. $^{\#\#}$P<0.05 vs. model group. TFR, total flavones of *Rhododendra* flower; CSE, cystathionine γ-lyase; ROCK, Rho-associated protein kinase.

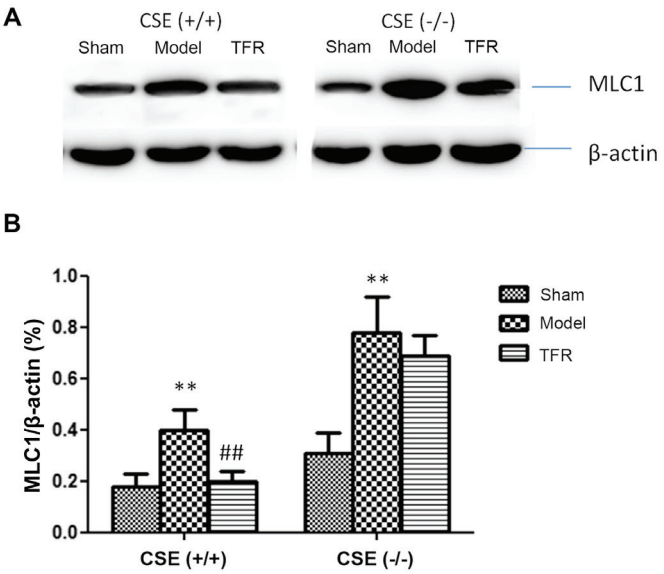

Figure 8. (A and B) Effect of TFR on the expression of MLC1 protein in isoproterenol-treated mice myocardium. **P<0.01 vs. sham group. $^{\#\#}$P<0.05 vs. model group. TFR, total flavones of *Rhododendra* flower; CSE, cystathionine γ-lyase; MLC1, membrane protein MCL1.

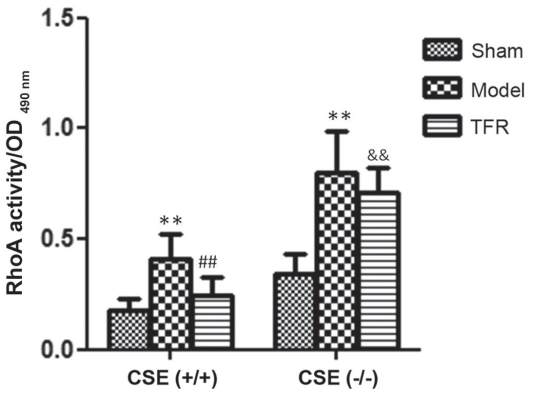

Figure 9. Effect of TFR on RhoA activity following A/R injury. **P<0.01 vs. sham. $^{\#\#}$P<0.01 vs. model. $^{\&\&}$P<0.01 vs. wild type TFR. TFR, total flavones of *Rhododendra* flower; CSE, cystathionine γ-lyase.

induced by ISO were markedly inhibited by TFR treatment. These results suggested that TFR had cardioprotective effects in myocardial ischemia that may be attributed to the inhibition of the RhoA/ROCK signal pathway.

Endogenous H_2S has been suggested as a novel signal transmitter and neuromodulator (36). In recent years, growing evidence has demonstrated that H_2S is a critical mediator of heart functions and serves a protective function in the pathogenesis and progress of heart diseases. Geng *et al* (37) identified that the CSE/H_2S pathway exists in the heart and has physiological effects such as negative inotropy and reduced central venous pressure. NaHS significantly decreased the infarct size of the left ventricle and mortality after acute MI in rats (38). In an additional study, sulfur dioxide (SO_2) preconditioning significantly decreased A/R induced myocardial injury *in vivo*, which is associated with increased myocardial antioxidative capacity and upregulated H2S/CSE pathway (39). It also has been revealed to protect against hyperglycemia-induced ROS-mediated apoptosis by upregulating the PI3K/AKT/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which subsequently activates Nrf2-regulated antioxidant enzymes in cardiomyocytes exposed to high glucose (40). However, the association between H_2S and the RhoA/ROCK signaling pathway remains unknown.

In the present study, it was identified that TFR-mediated inhibition of the RhoA/ROCK signal pathway may have been mediated by the CSE-H_2S axis. These results suggested that TFR exhibited cardioprotective effects in myocardial ischemia that may be attributed to an inhibition of the RhoA/ROCK signal pathway. The expression levels of ROCK1, ROCK2 and MLC1 induced by ISO in the KO mice were markedly increased compared with the WT mice. These results suggested that CSE KO led to decreased H_2S expression and activation of the RhoA/ROCK signal pathway and that H_2S had protective effects against A/R injury in mice hearts by inhibiting the RhoA/ROCK signal pathway.

In the present study, accompanying the pathophysiological process of ISO-induced myocardial injury was the impaired endogenous CSE/H_2S pathway. Administering exogenous H_2S resulted in effective protection of the myocytes and contractile activity by directly scavenging oxygen free radicals and decreasing the accumulation of lipid peroxidations. These results suggest that H_2S not only alleviated the pathological process of ischemic heart disease but may also serve as a cardiovascular protective regulator, and as a novel target in the prevention or treatment of cardiovascular diseases. TFR exhibited protective effects against A/R injury in mice hearts by inhibiting the RhoA/ROCK signal pathway and may have been mediated by the CSE-H_2S.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZC designed the experiment. YJ, YG and YL performed the experiment. YJ analyzed the data. YJ prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee for Animal Experiments of Anhui Medical University (no. 20160315).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Zhao K, Li H, Li S and Yang G: Regulation of cystathionine gamma-lyase/H_2S system and its pathological implication. Front Biosci (Landmark Ed) 19: 1355-1369, 2014.
2. Chang L, Geng B, Yu F, Zhao J, Jiang H, Du J and Tang C: Hydrogen sulfide inhibits myocardial injury induced by homocysteine in rats. Amino Acids 34: 573-585, 2008.
3. Sivarajah A, Collino M, Yasin M, Benetti E, Gallicchio M, Mazzon E, Cuzzocrea S, Fantozzi R and Thiemermann C: Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a mice model of regional myocardial I/R. Shock 31: 267-274, 2009.
4. Yang G, Li H, Tang G, Wu L, Zhao K, Cao Q, Xu C and Wang R: Increased neointimal formation in cystathionine gamma-lyase deficient mice: Role of hydrogen sulfide in α5β1-integrin and matrix metalloproteinase-2 expression in smooth muscle cells. J Mol Cell Cardiol 52: 677-688, 2012.
5. Mani S, Li H, Untereiner A, Wu L, Yang G, Austin RC, Dickhout JG, Lhoták Š, Meng QH and Wang R: Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. Circulation 127: 2523-2534, 2013.
6. Wang R: Two's company, three's a crowd: Can H2S be the third endogenous gaseous transmitter? FASEB J 16: 1792-1798, 2002.
7. Wang R: The gasotransmitter role of hydrogen sulfide. Antioxid Redox Signal 5: 493-501, 2003.
8. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, *et al*: H2S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. Science 322: 587-590, 2008.
9. Li H, Mani S, Cao W, Yang G, Lai C, Wu L and Wang R: Interaction of hydrogen sulfide and estrogen on the proliferation of vascular smooth muscle cells. PLoS One 7: e41614, 2012.
10. Loirand G, Guérin P and Pacaud P: Rho kinases in cardiovascular physiology and pathophysiology. Circ Res 98: 322-334, 2006.
11. Dong M, Yan BP, Liao JK, Lam YY, Yip GW and Yu CM: Rho-kinase inhibition: A novel therapeutic target for the treatment of cardiovascular diseases. Drug Discov Today 15: 622-629, 2010.
12. Satoh K, Fukumoto Y and Shimokawa H: Rho-kinase: Important new therapeutic target in cardiovascular diseases. Am J Physiol Heart Circ Physiol 301: H287-H296, 2011.
13. Shimokawa H and Satoh K: 2015 ATVB plenary lecture translational research on rho-kinase in cardiovascular medicine. Arterioscler Thromb Vasc Boil 35: 1756-1769, 2015.
14. Zhang J, Li XX, Bian HJ, Liu XB, Ji XP and Zhang Y: Inhibition of the activity of Rho kinase reduces cardiomyocyte apoptosis in heart ischemia/reperfusion via suppressing JNK-mediated AIF translocation. Clin Chim Acta 401: 76-80, 2009.
15. Li Y, Zhu W, Tao J, Xin P, Liu M, Li J and Wei M: Fasudil protects the heart against ischemia-reperfusion injury by attenuating endoplasmic reticulum stress and modulating SERCA activity: The differential role for PI3K/Akt and JAK2/STAT3 signaling pathways. PLoS One 7: e48115, 2012.
16. Dai SJ, Chen RY and Yu DQ: Studies on the flavonoid compounds of Rhododendron anthopogonoides. Zhongguo Zhong Yao Za Zhi 29: 44-47, 2004 (In Chinese).
17. Huang Y, Yin P, Jiang DF, Wang CY, Tan R, Wan L, Zhang Y and Fan G: Quality standard of rhododendron flos. World Sci Technol 16, 151-155,2014.
18. Yuan LP, Chen ZW, Li F, Dong LY and Chen FH: Protective effect of total flavones of Rhododendra on ischemic myocardial injury in rabbits. Am J Chin Med 34: 483-492, 2006 (In Chinese).
19. Zhang JH, Chen ZW and Wu Z: Late protective effect of pharmacological preconditioning with total flavones of Rhododendra against myocardial ischemia-reperfusion injury. Can J Physiol Pharmacol 86: 131-138, 2008.
20. Jiao Y, Fan YF, Wang YL, Zhang JY, Chen S and Chen ZW: Protective effect and mechanism of total flavones from rhododendron simsii Planch flower on cultured Rat cardiomyocytes with anoxia and reoxygenation. Evid Based Complement Alternat Med 2015: 863531, 2015.
21. Hausenloy DJ, Tsang A and Yellon DM: The reperfusion injury salvage kinase pathway: A common target for both ischemic preconditioning and postconditioning. Trends Cardiovasc Med 15: 69-75, 2005.

22. Terrell AM, Crisostomo PR, Wairiuko GM, Wang M, Morrell ED and Meldrum DR: Jak/STAT/SOCS signaling circuits and associated cytokine-mediated inflammation and hypertrophy in the heart. Shock 26: 226-234, 2006.
23. Demirynrek S, Kara AF, Celik A, Babül A, Tarakçioglu M and Demiryürek AT: Effects of fasudil. A Rho-kinase inhibitor. on myocardial preconditioning in anesthetized mice. Eur J Pharmacol 527: 129-140, 2005.
24. Janet C, Garber R, Wayne B, Joseph T, Bielitzki, Leigh AC, John C, Donovan, *et al*: Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. NIH Publication no. 85-23, revised, 2011.
25. Rona G, Chappel CI, Balazs T and Gaudry R: An infarct-like myocardial lesion and other toxic manifestations produced by isoproterenol in the rat. AMA Arch Pathol 67: 443-455, 1959.
26. Xu X, Li H, Gong Y, Zheng H and Zhao D: Hydrogen sulfide ameliorated lipopolysaccharide-induced acute lung injury by inhibiting autophagy through PI3K/Akt/mTOR pathway in mice. Biochem Biophys Res Commun 507: 514-518, 2018.
27. Chau VQ, Salloum FN, Hoke NN, Abbate A and Kukreja RC: Mitigation of the progression of heart failure with sildenafil involves inhibition of RhoA/Rho-kinase pathway. Am J Physiol Heart Circ Physiol 300: H2272-H2279,2011.
28. Yatani A, Irie K, Otani T, Abdellatif M and Wei L: RhoA GTPase regulates L-type Ca2+ currents in cardiac myocytes. Am J Physiol Heart Circ Physiol 288: H650-H659, 2005.
29. Shimokawa H, Hiramori K, Iinuma H, Hosoda S, Kishida H, Osada H, Katagiri T, Yamauchi K, Yui Y, Minamino T, *et al*: Anti-anginal effect of fasudil, a Rho-kinase inhibitor, in patients with stable effort angina: A multicenter study. J Cardiovasc Pharmacol 40: 751-761, 2002.
30. Saito Y, Kondo H and Hojo Y: Granzyme B as a novel factor involved in cardiovascular diseases. J Cardiol 57: 141-147, 2011.
31. Hamid SA, Bower HS and Baxter GF: Rho kinase activation plays a major role as a mediator of irreversible injury in reperfused myocardium. Am J Physiol Heart Circ Physiol 292: 2598-2606, 2007.
32. Hu Y, Chen X, Pan TT, Neo KL, Lee SW, Khin ES, Moore PK and Bian JS: Cardioprotection induced by hydrogen sulfide preconditioning involves activation of ERK and PI3K/Akt pathways. Pflugers Arch 455: 607-616, 2008.
33. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M and Narumiya S: Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 389: 990-994, 1997.
34. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, Hattori T, Nakashima Y, Kaibuchi K, Sueishi K and Takeshit A: Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. Circ Res 94: 385-393, 2004.
35. Chen ZW, Ma CG, Fang M and Xu SY: The blocking effect of hyperin on the inward flow of calcium ion. Yao Xue Xue Bao 29: 15-19, 1994 (In Chinese).
36. Kimura H: Hydrogen sulfide as a neuromodulator. Mol Neurobiol 26: 13-19, 2002.
37. Geng B, Yang J, Qi Y, Zhao J, Pang Y, Du J and Tang C: H2S generated by heart in rat and its effects on cardiac function. Biochem Biophys Res Commun 313: 362-368, 2004.
38. Zhu YZ, Wang ZJ, Ho P, Loke YY, Zhu YC, Huang SH, Tan CS, Whiteman M, Lu J and Moore PK: Hydrogen sulfide and its possible roles in myocardial ischemia in experimental rats. J Appl Physiol (1985) 102: 261-268, 2007.
39. Jin HF, Wang Y, Wang XB, Sun Y, Tang CS, and Du JB: Sulfur dioxide preconditioning increases antioxidative capacity in rat with myocardial ischemia reperfusion (I/R) injury. Nitric Oxide 32: 56-61, 2013.
40. Tsai CY, Wang CC, Lai TY, Tsu HN, Wang CH, Liang HY and Kuo WW: Antioxidant effects of diallyl trisulfide on high glucose-induced apoptosis are mediated by the PI3K/Akt-dependent activation of Nrf2 in cardiomyocytes. Int J Cardiol 168: 1286-1297, 2013.