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# RhoA/ROCK pathway regulates hypoxia-induced myocardial cell apoptosis

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ABSTRACT

**Objective:** To observe the regulatory effects of RhoA/ROCK pathway on the apoptosis of cardiac myocyte induced by anoxia and its mechanism. **Methods:** The model of cardiac myocyte anoxia was established. The beat pulsations and apoptosis rates after 1 h, 3 h, 6 h, 9 h and 12 h of anoxia were recorded and the expressions of RhoA, ROCK1/2, p-PI3K, p-AKT and caspae-3 were detected, too. The apoptosis and the expressions of related proteins were detected after RNAi of RhoA and the inhibition of ROCK by Y-27632. **Results:** The beat pulsations after 1 h, 3 h, 6 h, 9 h and 12 h decreased gradually but the apoptosis rates increased gradually, and the expressions of RhoA, ROCK1/2, p-PI3K, p-AKT and caspase-3 were increasing along with the increasing duration of anoxia. The apoptotic rates after 1 h, 3 h, 6 h, 9 h and 12 h of anoxia were (4.36±0.98)%, (8.36±2.12)%, (15.32±3.62)%, (18.68±4.83)% and (24.56±6.22)%, respectively and decreased more significantly than control group in different time points of anoxia ($P<0.05$), and the expressions of RhoA, ROCK1/2, p-PI3K, p-AKT and caspase-3 decreased significantly ($P<0.05$). The apoptosis rate and the expressions of RhoA, ROCK1/2, p-PI3K, p-AKT and caspase-3 decreased significantly ($P<0.05$) after the inhibition of ROCK by Y-27632 ($P<0.05$). **Conclusions:** RhoA/ROCK pathway plays a critical role in the regulation of the apoptosis of cardiac myocyte induced by anoxia, which may be accompanied by regulating the activity of PI3K/AKT/Caspase-3 pathway.

## 1. Introduction

Rho (Ras homologue) proteins are small-molecule proteins with GTPase activity, and they play important biological roles in cell division and proliferation[1]. RhoA is a small G protein which has been investigated more extensively than any other members of the Rho family. RhoA regulates downstream factors, changes the structure of actin, and subsequently regulates the morphology, proliferation, apoptosis, adhesion, metastasis as well as other biological behaviors of cells. ROCK (Rho associated coil-coil protein kinase) is a recently discovered kinase that is associated with the apoptosis of cells[2]. ROCK is an important effector of RhoA. It has been demonstrated recently that the RhoA/ROCK pathway is associated with coronary atherosclerosis[3,4], myocardial ischemia/reperfusion injury[5], myocardial hypertrophy[6], and myocardial fibrosis[7]. ROCK was found to play an important regulatory role in myocardial apoptosis and hyperplasia[8-11]. In this study, a model for hypoxia-induced myocardial apoptosis was established, and changes in RhoA and ROCK expressions with the apoptosis were observed. To observe the effect of suppressing the RhoA /ROCK pathway on hypoxia-induced myocardial apoptosis and the effect of blocking the RhoA /ROCK pathway on PI3K/AKT/caspase-3 pathway, RNA interference (RNAi) was conducted to suppress RhoA activity and Y-27632 was used to suppress ROCK activity.

## 2. Materials and methods

### 2.1. Myocardial cell culture

Myocardial cells were harvested from 1-3 d old neonatal rats. In brief, the rat heart tissue was digested three times

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with 0.08% trypsin, and trypsin was then removed by centrifugation. Myocardial tissue was digested with 0.01% type II collagenase for 2 h, followed by addition of 15% fetal bovine serum-containing DMEM medium to terminate digestion. The cells were collected by 5 min centrifugation at room temperature, and resuspended in 15% fetal bovine serum-containing DMEM medium. The filtered cell suspension was transferred into culture dishes, and cultured for 2 h in a $CO_2$-containing incubator. In order to suppress fibroblast growth, 0.1 mM 5′-Brdu was added. Upon cell confluence and synchronized pulsation, DMEM medium was added to prepare cell suspension which was stored in liquid nitrogen prior to use.

### 2.2. Preparation of myocardial cell hypoxia model

A solution to mimic ischemia was prepared, which contained 0.9 mmol/L $NaH_2PO_4$, 6.0 mmol/L $NaHCO_3$, 1.8 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 20 mmol/L HEPES, 98.5 mmol/L NaCl, 10.0 mmol/L KCl, and 40 mmol/L sodium lactate, pH 6.5. The solution was pretreated with 95% $N_2$-5% $CO_2$ for 1 h and then substituted with DMEM medium for hypoxic myocardial cells. In control group, myocardial cells were cultured with DMEM medium.

### 2.3. RhoA RNA interference plasmid construction and myocardial cell transfection

According to the design principles of short hairpin RNA (shRNA) sequences, RhoA interference sequences designed for RhoA mRNA sequence registered in NCBI Gene Bank (gene ID: NM_001664) were as follows: sense 5′-TTTCTAAACTATCAGGGCTG-3′; antisense 5′-GCCCTGATAGTTTAGAAAAT-3′. A negative control sequences were designed: sense 5′-CAGT-CAGGAGGATCCAAAGTG-3′, antisense 3′-TTGTCAGTCCTCCTAGGTTTC-5′. There was a loop between the sense and antisense strands. To the 5′-end of the sense strand added a BamH I restriction site, and to its 3′-end added a termination codon. To the 5′-end of the antisense strand added a HindIII restriction site. The sequences were synthesized by Takara Bio (Dalian, China). The synthetic oligonucleotide strands were annealed to form dsDNA and cloned into the linearized vector pRNAT-U6.1/neo. The resulting plasmids were termed pRNAT-U6.1/neo/RhoA/shRNA, and pRNAT-U6.1/neo/RhoA/shRNA-N, respectively. The plasmids were transfected into competent *Escherichia coli* DH5 α, and then the bacteria were inoculated into culture dishes with 100 μg/mL ampicillin containing LB medium. The single positive cloneswere subjected to amplified culture. The plasmids were extracted and sequenced. The plasmids were transfected into myocardial cells using liposome 2000. There were an interference group (transfected with pRNAT-U6.1/neo/RhoA/shRNA-1), a negative control group (transfected with pRNAT-U6.1/neo/RhoA/shRNA-N), and a blank control group (transfected with void vector). The cells were screened for around 2 weeks with the use of G418 until single cell clones appeared. Under fluorescence microscopy, single cell clones that emitted green fluorescence were picked and subcultured. RhoA protein expression was detected by Western blot in each group.

### 2.4. Treatment with ROCK inhibitor (Y-27632)

In treatment group, myocardial cells were cultured with DMEM medium containing 20 μmol/L Y-27362. In control group, isovolumetric PBS was added in place of Y-27362 solution. The apoptosis rate and protein expressions were observed in each group at various time points during hypoxia.

### 2.5. Apoptosis assays

Apoptosis was detected by flow cytometry using annexin V-FITC/PI apoptosis assay kits (KeyGEN Biotech, Nanjing, China). According to the kit's instructions, the detection was carried out as described below. Cell suspension was prepared by enzymatic digestion, and centrifuged. After removal of the supernatant, cells were resuspended in 195 μL Annexin V-FITC binding solution, and then added with 5 μL Annexin V-FITC, followed by 10 min incubation at room temperature in the dark. The cell suspension was centrifuged, and the supernatant was removed. Then, cells were resuspended in 190 μL Annexin V-FITC binding solution, and added with 10 μL PI staining solution, which was immediately followed by flow cytometry. Viable cells were resistant to staining with Annexin V-FITC and PI; cells at early stages of the apoptosis were stained with Annexin V-FITC, but not with PI; necrotic cells and cells at late stages of the apoptosis were stained with both Annexin V-FITC and PI.

### 2.6. Western Blot

Goat anti-human RhoA, ROCK 1/2, p-PI3K, p-AKT and caspase 3 polyclonal antibodies were purchased from Santa Cruz (USA). Murine anti-human β-actin, horseradish peroxidase (HRP) conjugated goat anti-murine secondary antibodies and rabbit anti-goat secondary antibodies were purchased from Boster (China). Cells at logarithmic growth phase were lyzed with RIPA protein lysis buffer, followed by 5 min centrifugation (4 ℃, 10 000 r/min, radius 4 cm). The supernatants were harvested and determined for protein concentration with BCA method. 50 μg protein was added with 2×loading buffer, denatured at 100 ℃ for 5 min, and subjected to SDS-PAGE. The protein was then transferred onto a nitrocellulose filter, incubated with specific primary antibodies and secondary antibodies, colored by enhanced chemiluminescence (ECL) (Boster, China). After X-ray

exposure, development and fixing, the autoradiographs were scanned with BandScan software for grayscale analysis.

### 2.7. Statistical analysis

Data were expressed as mean±SD. Paired data were subjected two-sided *t*-test using SPSS16.0, and $P<0.05$ was deemed as statistically significant difference.

## 3. Results

### 3.1. During hypoxia myocardial cell pulsation frequency decreased gradually and the apoptosis rate increased gradually

Cell pulsation frequency and the apoptosis rate were observed in the myocardial cell hypoxia model after 1 h, 3 h, 6 h, 9 h, and 12 h of hypoxia. The results showed that cell pulsation frequency decreased gradually with the increasing duration of hypoxia, and decreased significantly after 3 h of hypoxia when compared to before hypoxia ($P<0.05$). After 12 h of hypoxia, only a few cells were pulsatile, with the mean pulsation frequency reduced to (32±5) beats/min, a figure significantly lower than that before hypoxia ($P<0.01$). Flow cytometry indicated that the apoptosis increased gradually with the increasing duration of hypoxia, and the apoptosis rate was significantly higher after 1 h of hypoxia than before hypoxia ($P<0.01$), and reached approximately 60% after 12 h of hypoxia (Table 1).

**Table 1**
Pulsation frequencies and the apoptosis rates of myocardial cells during hypoxia.

| Groups | Beat frequency (times/Sec) | Cell apoptosis (%) |
|---|---|---|
| Control group | 186±24 | 1.26±0.16 |
| After 1 h of anoxia | 170±15 | 8.32±1.21** |
| After 3 h of anoxia | 152±10* | 18.26±3.21** |
| After 6 h of anoxia | 118±12** | 28.46±3.28** |
| After 9 h of anoxia | 82±8** | 43.68±6.52** |
| After 12 h of anoxia | 32±5** | 58.93±7.28** |

Compared with control group, *$P<0.05$, or **$P<0.01$.

### 3.2. Expressions of RhoA, ROCK 1/2, p-PI3K, p-AKT, and caspase-3 increased in myocardial cells during hypoxia

Western blotting showed that RhoA protein expression increased significantly after 1 h of hypoxia (0.38±0.07) when compared with before hypoxia (0.12±0.03) ($P<0.05$). With the increasing duration of hypoxia, RhoA protein expression increased gradually, and reached a peak (1.28±0.26) after 9 h of hypoxia. Meanwhile, ROCK 1/2 expression increased gradually with the increasing duration of hypoxia, and reached a peak after 9h hypoxia (0.68±0.21, 0.52±0.16), which were significantly higher than the levels before hypoxia (0.18 ±0.03, 0.12±0.03) ($P<0.01$). PI3K/AKT/caspase-3 expressions also increased gradually with the duration of hypoxia increasing (Figure 1).

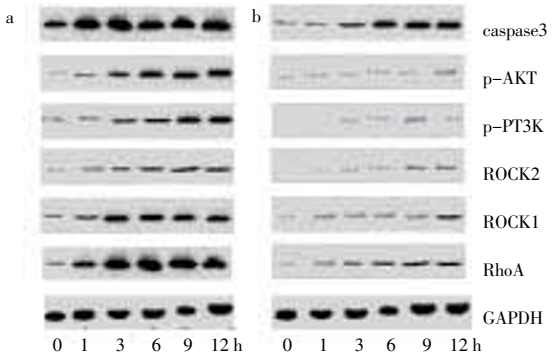


**Figure 1.** Western blot detection of the expressions of RhoA, ROCK 1/2, p-PI3K, p-AKT and caspase-3.
a: the expressions of RhoA, ROCK 1/2, p-PI3K, p-AKT and caspase-3 increased gradually in myocardial cells during hypoxia. b: RhoA interference suppressed the expressions of ROCK 1/2, p-PI3K, p-AKT and caspase-3.

### 3.3. RhoA interference suppressed the expressions of ROCK 1/2, p-PI3K, p-AKT and caspase-3

A hypoxia model was prepared with cells treated with RhoA interference, and protein expressions were detected at the time points of 1 h, 3 h, 6 h, 9 h, and 12 h of hypoxia. The results showed that after interference, RhoA expressions downregulated significantly at different time points during hypoxia when compared with control group ($P<0.01$). Meanwhile, in the expressions of ROCK 1/2 andp-PI3K/p-AKT/caspase-3 were significantly lower than those incontrol group at different time points during hypoxia due to downregulated expression of RhoA ($P<0.05$) (Figure 2).

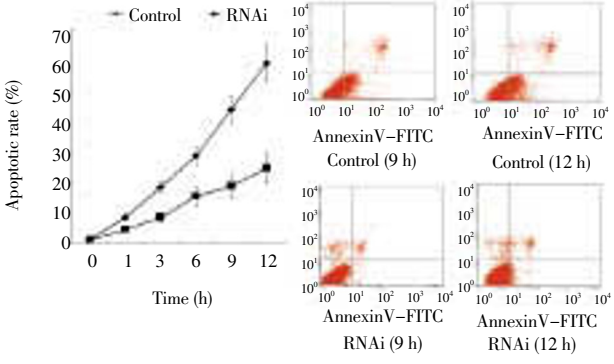


**Figure 2.** Following RhoA interference, the apoptosis rates were significantly lower than those in control group at time points of 1 h, 3 h, 6 h, 9 h and 12 h of hypoxia.

### 3.4. RhoA interference suppressed hypoxia-induced myocardial apoptosis

Following RhoA interference, the apoptosis rates were (4.36 ±0.98)%, (8.36±2.12)%, (15.32±3.62)%, (18.68±4.83)%, (24.56± 6.22)% at the time points of 1 h, 3 h, 6 h, 9 h, 12 h of hypoxia, respectively, which were significantly lower than the figures in control group at the corresponding time points ($P<0.05$)

(Figure 3).

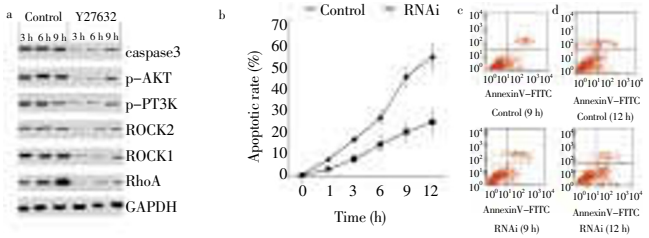


**Figure 3.** Blockage of the ROCK pathway suppressed myocardial apoptosis and PI3K/AKT/caspase-3 pathway.
a: Western blot showed that Y-27632 suppressed the expressions of RhoA, PI3K/AKT/caspase-3 in myocardial cells during hypoxia. b: Y27632 decreased the myocardial apoptosis rate. c: Flow cytometry revealed the apoptosis rates in the group treated with Y-27632 at 9 h and 12 h of hypoxia. D: Flow cytometry revealed the apoptosis rates in control group at 9 h and 12 h of hypoxia.

### *3.5. Blockage of the ROCK pathway suppressed myocardial apoptosis and PI3K/AKT/caspase-3 pathway*

After Y-27632 suppression of ROCK expression, RhoA and PI3K/AKT/caspase-3 levels in the myocardial cells were significantly lower than those in the control group at various time points during hypoxia. Apoptosis was detected following Y27632 treatment of cells. The results showed that the apoptosis rates of myocardial cells were (3.62±0.64)%, (8.21±1.16)%, (14.56±3.48)%, (20.21±4.96)%, (24.56±5.89)% at 1 h, 3 h, 6 h, 9 h, 12 h of hypoxia, respectively, which were significantly lower than the rates in the control group at corresponding time points ($P$<0.05).

## 4. Discussion

ROCK, which belongs to the protein kinase family, acts as an important effector of the Rho family. ROCK 1 is a RhoA associated serine/threonine kinase, and its function as a downstream effector of RhoA has been investigated in depth. ROCK receives activating signals from RhoA, becomes activated after phosphorylation of multiple amino acid sites, and mediates a series of downstream phosphorylation dephosphorylation events. Hence, it is an important kinase which regulates cell mobility. ROCK protein mainly alters cytoskeletal structure and cell polarity and affects actin stability and myosin contractility[12-14]. Moreover, there exists a reciprocal feedback mechanism among cell adhesiveness, cytoskeleton and RhoA /ROCK[15]. RhoA/ROCK plays important pathophysiologic roles in cell hyperplasia, survival, apoptosis, mobility, migration and tumorigenesis[16,17]. Current research on the RhoA /ROCK pathway mainly focuses on its suppression of invasiveness and metastasis of malignant cells, which is promising in discovery of therapeutic targets of malignancies[18]. Over recent years, the role of RhoA /ROCK pathway in cardiovascular diseases has been stressed. The RhoA /ROCK pathway was found to relate to atherosclerosis, hypertension, pulmonary hypertension, myocardial infarction, myocardial hypertrophy, and heart failure. It was reported that pressure overload may lead to rapid activation of ROCK in rat myocardial cells[19]. In regional myocardial ischemia/reperfusion injury, myocardial RhoA expression and ROCK activity all increased[19,20]. In order to investigate the roles of RhoA/ROCK in myocardial apoptosis, a hypoxic myocardial cell model was established. We observed myocardial cell apoptosis during hypoxia, while RhoA and ROCK protein expression increasing proportionally. These findings suggest the role of RhoA/ROCK in hypoxia-induced myocardial apoptosis.

In order to further investigate the role of the RhoA/ROCK pathway in myocardial apoptosis, we downregulated RhoA expression by myocardial cells through RNAi and observed the effect of RhoA suppression on apoptosis and ROCK protein expression. The results showed that RhoA interference led to ROCK 1/2 expression downregulation, and that the apoptosis rate of myocardial cells decreased significantly during hypoxia. In addition, we used the specific ROCK inhibitor Y-27632 to treat myocardial cells so as to block the ROCK pathway. The results showed that Y-27632 suppressed hypoxia-induced myocardial apoptosis. ROCK inhibitors may improve ventricular hypertrophy and heart function in hypertensive rats[21-23], and ROCK activity is associated with left ventricular remodeling after myocardial infarction and myocardial fibrosis[24]. Administration of ROCK inhibitors at early stages of ischemia/reperfusion may significantly diminish myocardial infarct areas and the myocardial apoptosis rate in Sprague-Dawley rats[25].

In summary, RhoA /ROCK play an important regulatory role in hypoxia-induced myocardial apoptosis. To investigate the underlying molecular mechanisms, we focused on the PI3K/AKT/caspase-3 signal transduction pathway. Recently, this pathway has been found to regulate cell proliferation, division, differentiation, and apoptosis[26,27]. It has been shown that the PI3K/AKT signal pathway is activated in ischemic preconditioning, and ischemia/reperfusion injury, suppressing myocardial apoptosis and exerting myocardial protection[28,29]. Ser473 and/or Thr308 phosphorylation is prerequisite for AKT activation, while AKT activation is prerequisite for its promotion of cell survival. AKT activation results in extensive biological effects, such as anti-apoptosis and promotion of cell survival, mainly through promoting phosphorylation of downstream substrates such as caspase 3. We found that the apoptosis rate of myocardial cells increased gradually with the increasing duration of hypoxia, which was accompanied by the activation of the RhoA / ROCK pathway and enhancement in PI3K/AKT/caspase-3 activity. RNA interference suppressed RhoA activity, resulting in suppression of the apoptosis and decrease in PI3K/AKT/caspase-3 activity. In addition, suppression of ROCK activity by a ROCK specific inhibitor inhibit PI3K/AKT/caspase-3 activity. These findings demonstrated that RhoA /ROCK may regulate the apoptosis through regulating the activity of PI3K/AKT/caspase-3 pathway.

The RhoA /ROCK pathway is activated in hypoxia-induced

myocardial apoptosis, and suppressing the RhoA /ROCK pathway may suppress myocardial apoptosis. The RhoA / ROCK pathway regulates hypoxiainduced myocardial apoptosis, possibly through regulating the activity of PI3K/ AKT/caspase-3 pathway.

## Conflict of interest statement

All the authors have no conflict of interest.

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