ROCK-dependent ATP5D modulation contributes to the protection of notoginsenoside NR1 against ischemia-reperfusion-induced myocardial injury

Ke He, Li Yan, Chun-Shui Pan, Yu-Ying Liu, Yuan-Chen Cui, Bai-He Hu, Xin Chang, Quan Li, Kai Sun, Xiao-Wei Mao, Jing-Yu Fan, and Jing-Yan Han

1Department of Integration of Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, Beijing, China; 2Tasly Microcirculation Research Center, Peking University Health Science Center, Beijing, China; 3Key Laboratory of Microcirculation, State Administration of Traditional Chinese Medicine of the People’s Republic of China, Beijing, China; and 4 Key Laboratory of Stasis and Phlegm, State Administration of Traditional Chinese Medicine of the People’s Republic of China, Beijing, China

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He K, Yan L, Pan CS, Liu YY, Cui YC, Hu BH, Chang X, Li Q, Sun K, Mao XW, Fan JY, Han JY. ROCK-dependent ATP5D modulation contributes to the protection of notoginsenoside NR1 against ischemia-reperfusion-induced myocardial injury. Am J Physiol Heart Circ Physiol 307: H1764–H1776, 2014. First published October 10, 2014; doi:10.1152/ajpheart.00259.2014.—Cardiac ischemia-reperfusion (I/R) injury remains a challenge for clinicians, which initiates with energy metabolism disorder. The present study was designed to investigate the protective effect of notoginsenoside R1 (NR1) on I/R-induced cardiac injury and underlying mechanism. Male Sprague-Dawley rats were subjected to 30-min occlusion of the left coronary anterior descending artery followed by reperfusion with or without NR1 pretreatment (5 mg·kg⁻¹·h⁻¹). In vitro, H9c2 cells were cultured under oxygen and glucose deprivation/reoxygenation conditions after NR1 (0.1 mM), Rho kinase (ROCK) inhibitor Y-27632 (10 μM), or RhOA/ROCK activator U-46619 (10 nM) administration. Myofibril and mitochondria morphology were observed by transmission electron microscopy. F-actin and apoptosis were determined by immunofluorescence and TUNEL staining. ATP and AMP content were assessed by ELISA. Phosphorylated-AMP-activated protein kinase, ATP synthase subunits, apoptosis-related molecules, and the level and activity of ROCK were determined by Western blot analysis. We found that NR1 pretreatment ameliorated myocardial infarction, histological injury, and cardiac function induced by I/R. Furthermore, similar to the effect of Y-27632, NR1 improved H9c2 cell viability, maintained actin skeleton and mitochondria morphology, and attenuated apoptosis induced by oxygen and glucose deprivation/reoxygenation. Importantly, NR1 prevented energy abnormality, inhibited the expression and activation of ROCK, and restored the expression of the mitochondrial ATP synthase β-subunit both in vivo and in vitro, whereas U-46619 suppressed the effect of NR1. These results prove NR1 as an agent able to prevent I/R-induced energy metabolism disorder via inhibiting ROCK and enhancing mitochondrial ATP synthase β-subunits, which at least partially contributes to its protection against cardiac I/R injury.

Notoginsenoside R1; myocardium ischemia-reperfusion injury; apoptosis; Rho kinase; inhibition; energy deficit

ISCHEMIC HEART DISEASE is among the top causes of death in the world. Percutaneous coronary intervention (PCI) has currently been applied widely to deal with acute coronary syndrome, myocardial infarction, and stable angina. Although PCI can restore blood flow in the myocardium rapidly, it does not reduce the risk of serious heart events because of reperfusion injury (2, 8). Thus, strategies to prevent reperfusion injury and improve PCI outcomes are currently appealing in the clinic.

Ischemia-reperfusion (I/R) injury starts with ischemia, which results in hypoxia and malnutrition, leading to energy metabolism disorder characterized by ATP deficiency. Energy metabolism disorder has been noted in myocardial I/R injury and used as a parameter for the assessment of cardiac damage after heart I/R (19, 32). The majority of ATP is produced by ATP synthase located in mitochondria, which consists of two regions and a total of eight subunits, including the mitochondrial ATP synthase β-subunit (ATP5D). Lack of ATP impacts a range of ATP-consuming processes, including cation pumps and F-actin polymerization; meanwhile, the degradation of ATP takes part in ROS production via the xanthine oxidase system. The cation pump disorder and excessive ROS drive cells toward death, whereas disarrangement of F-actin causes restrained systolic function (21, 23). Thus, maintenance of energy balance and improvement of ATP production are expected to be promising strategies for protection against cardiac I/R injury.

Rho kinase (ROCK) belongs to the AGC (PKA/PKG/PKC) family of serine/threonine protein kinases and is best known for mediating actin filament stabilization and the generation of actin-myosin contractility by phosphorylation of myosin phosphatase target subunit 1 (MYPT1) (35). The deleterious role of ROCK has been implicated in the progression of cardiovascular diseases in diverse ways (26). Up-to-date progress in translational research supports the notion that ROCK is an important therapeutic target for the treatment of various cardiovascular diseases, including ischemic damage. In cardiac I/R injury, ROCK inhibition with inhibitor such as Y-27632 resulted in reduced infarct size, less inflammation, reduced apoptosis, and enhanced contractile function (1, 11, 27). However, no study has been published as yet regarding whether ROCK is implicated in I/R-induced energy metabolism disorder and, if so, what the underlying mechanism is.

Notoginsenoside R1 (NR1) is a major effective ingredient of Panax notoginseng and has been reported to attenuate lipopolysaccharide-induced injury in the mouse heart (29). Previous studies (5, 17, 20) have demonstrated that NR1 could protect the rat liver, kidney, and intestine from I/R injury through anti-inflammatory and antiapoptosis. Red ginseng and ginsenoside Rd have been reported to have the potential to combat...
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Cardiac I/R injury (18a, 34a). However, the effect of NR1 on cardiac I/R injury still needs to be explored; specifically, the role of NR1 in the attenuation of I/R-induced cardiac energy deficiency is unknown.

The present study was conducted in a rat cardiac I/R model and a H9c2 cell oxygen and glucose deprivation/reoxygenation (OGD/R) model to investigate the role of NR1 in protecting myocardial structure and function from damage by I/R challenge, with a particular focus on the involvement of ROCK and ATP5D regulation in the effect of NR1 on energy metabolism disorder.

MATERIALS AND METHODS

Animals and the I/R model. Male Sprague-Dawley rats (weight: 240–260 g) were purchased from the Animal Center of Peking University (certificate no. SCXX (Jing) 2006-0008). Rats were housed at 22 ± 2°C and a relative humidity of 40 ± 5% under a 12:12-h light-dark cycle and given standard laboratory diet and water. Rats were fasted for 12 h before experiments but allowed to access water freely. These investigations conformed with European Union Adopted Directive 2010/63/EU and the Guide of the Peking University Animal Research Committee. The experimental protocol was approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010-001).

Rats were anesthetized with 2% pentobarbital sodium (60 mg/kg) by peritoneal injection and placed in a supine position. A thoracotomy was performed by removing the left third rib to expose the heart, and a 5-0 suture silk was placed around the left anterior descending coronary artery, which was 1–2 mm under the boundary of the pulmonary conus and left auricle. The suture silk was fastened for 30 min for the induction of ischemia and then released, allowing reperfusion (19). Animals in sham-operated (sham) group and NR1 treatment groups were fastened for 30 min before ischemia, animals were infused continuously (1 ml/h) with physiological saline (in the sham group and I/R group) or NR1 (5 mg·kg⁻¹·h⁻¹, Feng-Shan-Jian Medical, Kunming, China) solution in saline (in NR1 + sham group and NR1 + I/R group) via the femoral vein until the end of the experiment. A total of 168 rats were included and randomly assigned to different groups (see Table 1 for further details).

Cell culture. H9c2 cells (a rat cardiac myoblast cell line, American Type Culture Collection), used in vitro for determining underlying mechanisms (12), were cultured in DMEM (Invitrogen, Grand Island, UK) containing 4 mM l-glutamine, 4.5 g/l glucose, and 10% FBS (Invitrogen) at 37°C in a humidified incubator with 95% air and 5% CO2.

After medium was replaced with glucose-free DMEM without FBS, H9c2 cells were cultured for 15 h under OGD/R conditions in a humidified atmosphere of 5% CO₂ and 1% O₂. At the onset of hypoxia, cells were assigned to exposure to one of the following treatments: vehicle (OGD/R group), 10 μM Y-27632 (Y-27632 + OGD/R group), or 0.1 mM NR1 without (NR1 + OGD/R group) or with 10 nM U-46619 (NR1 + U-46619 + OGD/R group). After hypoxia, cells from the above four groups were furnished with fresh DMEM with glucose and FBS and rapidly transferred into a normoxic incubator for 24-h reoxygenation. The control group and NR1 + control group were subjected to normal incubation.

Myocardial infarct size. At 90 min after reperfusion, the anterior descending coronary artery was ligated, and 2 ml of 0.35% Evans blue were administered through the femoral vein. Hearts were rapidly excised and sliced parallel to the atrioventricular groove into five sections (each 1 mm thick) from the apex cordis to the ligation site. Slices were incubated for 15 min at 37°C in a 0.375% solution of 2,3,5-triphenyltetrazolium chloride in PBS and then photographed with a stereoscope connected with Digital sight DS-5M-U1 (NIKON, Nanjing, China). The infarction zone was stained white, the noninfarction zone was shown as blue, and the area at risk (AAR) was pink. The myocardial infarct size, AAR, and left ventricular (LV) size of each slice were analyzed by Image-Pro Plus 6.0 (Media Cybernetic, Bethesda, MD). Ratios of the AAR to LV (in %) and infarct area to AAR (in %) were calculated, and values from five slices were averaged and used to express the degree of myocardial infarction (38).

Myocardial histology. At 90 min after reperfusion, the heart was fixed in 4% paraformaldehyde (PFA) solution for 48 h and processed for paraffin sections. Paraffin sections (5 μm) were stained with hematoxylin and eosin, and photomicroscopy was then performed at ×200 magnification (19).

Heart function test. A cannula was inserted into the LV through the right carotid artery, which was connected to a biofunction experiment system (BL-420F, Chengdu Taimen Technology, Chengdu, China). LV systolic pressure (LVSVP), LV diastolic pressure (LVDP), LV maximum upstroke velocity (+dP/dmax), and LV maximum descent velocity (−dP/dmax) were measured with BL-420F equipment at baseline, 30 min after ischemia, and 30, 60, and 90 min after reperfusion.

Ultrastructure examination. At 90 min after reperfusion, the rat heart was perfused for 40 min with a fixative made of 4% PFA and 2% glutaraldehyde in 0.1 mol/l phosphate buffer at a speed of 3 ml/min, and alcohol (1-3 mm³ fresh myocardial tissue blocks were cut from the surrounding infarct region of the LV. Tissue blocks and pelleted H9c2 cells were fixed with 3% glutaraldehyde, incubated overnight at 4°C, and then postfixed with 1% osmium tetroxide for 2 h in the dark. After undergoing dehydration with a graded series of acetone, samples were preembedded in a mixture of acetone and Epon812 resin at 37°C overnight and embedded in embedding medium for 48 h at 60°C. Ultrathin sections were prepared as routine, stained with uranyl acetate and lead citrate, and then observed and photographed with a transmission electron microscope (JEOL, Tokyo, Japan).

Apoptosis assay. Myocardial tissue and H9c2 cells were fixed in 4% PFA. Myocardial sections for double staining of α-smooth muscle actin and TUNEL were incubated with a mixture of acetone and Epon812 resin at 37°C overnight and then postfixed with 1% osmium tetroxide for 2 h in the dark. After undergoing dehydration with a graded series of acetone, samples were preembedded in a mixture of acetone and Epon812 resin at 37°C overnight and embedded in embedding medium for 48 h at 60°C. Ultrathin sections were prepared as routine, stained with uranyl acetate and lead citrate, and then observed and photographed with a transmission electron microscope (JEOL, Tokyo, Japan).

Table 1. Numbers of animals for the different experimental groups and various parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham Group</th>
<th>NR1 + Sham Group</th>
<th>I/R Group</th>
<th>NR1 + I/R Group</th>
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<td>8</td>
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The same animals were used for hematoxylin and eosin staining, TUNEL assay, and F-actin staining. Sham, sham operation; NR1, notoginsenoside R1 pretreatment; I/R, ischemia-reperfusion.
calculated and expressed as a percentage of TUNEL-positive cardiomyocytes (19).

Cell viability and lactate dehydrogenase assay. H9c2 cell viability was determined colorimetrically using Cell Counting Kit-8 assay according to the manufacturer’s instructions (DOJINDO, Kumamoto, Japan). The release of lactate dehydrogenase (LDH) into the incubation medium was measured using a rat LDH ELISA Kit (R&D, Minneapolis, MN) by a microplate reader.

Immunofluorescence staining and confocal microscopy. Fixed cardiac paraffin sections and H9c2 cells were permeabilized with 0.3% Triton X-100, blocked in goat serum, and then stained with rhodamine phalloidin (Invitrogen, Carlsbad, CA) for F-actin at 37°C for 2 h. Cells were incubated with anti-ROCK1 and anti-phosphorylated (p-)MYPT1 antibodies (Invitrogen) for 12 h at 4°C. Dylight 488-labeled anti-rabbit secondary antibody was applied for 1 h in the dark at 37°C. Nuclei were labeled with Hoechst 33342. Immunofluorescence staining was examined by a laser scanning confocal microscope (TCS SP5, Mannheim, Germany).

Assessment of ATP and AMP. Rats were perfused with saline under anesthesia, and hearts were removed. Tissue from the LV was sampled at ~2 mm under the ligature. H9c2 cells were harvested and mixed in 100 μl RIPA lysis buffer. Whole protein from tissue and cells was extracted with a protein extraction kit (Applygen, Beijing, China). In brief, the mixture was homogenized, incubated on ice for 30 min, and centrifuged at 20,000 g and 4°C for 10 min. The resultant supernatant was taken as whole protein. The content of ATP and AMP of the myocardium was assessed by ELISA and detected by a microplate reader (Multiskan MK3, Thermo) according to manufacturer’s instructions (17, 19).

ATP synthase activity. H9c2 cells were harvested and then pipetted up and down to break apart clumps of cells. The sample was frozen, thawed, and then pelleted at 20,000 g to fracture membranes. Total protein concentration was detected with a BCA protein assay kit (Applygen) and adjusted to a concentration of 5.5 mg/ml. The sample was added with detergent at a ratio 1:10 (vol/vol), mixed, and then incubated on ice for 30 min. After centrifugation at 20,000 g for 20 min, the supernatant was collected. ATP synthase activity was determined using an ATP Synthase Enzyme Activity Microplate Assay Kit (Abcam), with the plate set in the MULTISKAN MK3 enzyme microplate reader (Thermo Fisher Scientific). The absorbance of each well was measured at 340 nm and 30°C for 60 min using a kinetic program. The activity rate was expressed as the change in absorbance at 340 nm (in optical density units/min).

Western blot analysis. Whole protein was extracted as described above. The concentration of whole protein was determined with a BCA protein assay kit (Applygen) according to the manufacturer’s instructions. Samples were separated by SDS-PAGE and transferred to polyvinylidine difluoride membranes. Primary antibodies used were those against p-AMP-activated protein kinase (p-AMPK; CST), ATP synthase-α and -β (BD), ATP5D (Santa Cruz Biotechnology), β-actin (CST), Bcl-2 (CST), Bax (CST), caspase-3 (no. 9662, CST), ROCK1 (Invitrogen), and MYPT1/p-MYPT1 (Invitrogen). Horseradish peroxidase-linked secondary antibody was used to visualize bound primary antibodies with chemiluminescence substrate (Applygen). Protein was quantified by scanning densitometry in the X-film using Image-Pro plus 6.0 (Media Cybernetics). For quantification, band intensity was assessed via Bio-Rad Quantity One software.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed using one-way ANOVA followed by a Newman-Keuls test or using two-way ANOVA followed by a Bonferroni test for multiple comparisons (LVSP, LVDP, +dP/dt max, and −dP/dt max). Data were analyzed using GraphPad Prism 5 software (GraphPad Software). P values of <0.05 were considered to be statistically significant.

RESULTS

NRI diminishes I/R-induced myocardial infarct size and tissue injury. Myocardial infarcts were assessed by Evans blue-2,3,5-triphenyltetrazolium chloride staining after 90 min of reperfusion, and representative heart slices from the different groups are shown in Fig. 1A, where the pink area represents ischemic myocardial tissue, whereas the white area represents the infarction region. Apparently, myocardial tissue slices from the sham group and NRI + sham group exhibited no infarct. In contrast, noticeable infarct areas were observed in myocardial tissue slices in the I/R group. Compared with the I/R group, heart slices from NRI pretreatment groups had obviously smaller infarct areas but similar areas of ischemic regions. This impression was corroborated by quantitative analysis of AAR/LV and infarct area/AAR, as shown in Fig. 1, B and C, respectively. Ninety minutes after reperfusion, AAR/LV obviously increased in the I/R group compared with the sham group, but there was no significant difference found between I/R and NR1 + I/R groups. Infarct area/AAR in the I/R group increased proportionally as well compared with the sham group, which, however, was attenuated significantly by pretreatment with NR1. These results demonstrate the protection of NR1 on I/R-induced myocardial infarct.

Compared with the sham group, distinct alterations occurred in the surroundings of infarction areas of myocardial tissues from the I/R group (Fig. 2A), including myocardial interstitial edema, rupture of myocardial fibers, and infiltration of leukocytes. However, pretreatment with NR1 ameliorated the I/R-induced myocardial alterations, particularly the interstitial edema and myocardial fiber disruption. F-actin stained with rhodamine phalloidin in the I/R group showed severe rupture and abundant actin bundle formation (Fig. 2B). Apparently, the I/R-induced F-actin rearrangement was alleviated in the NR1 group. Representative ultrastructural images of the myocardium from the different groups are shown in Fig. 2C. Of note, the myocardium in the sham group displayed a normal ultrastructure with regularly myofibrils, well-preserved sarcomeres, and densely packed mitochondria. I/R challenge provoked a dramatic injury in myocardial ultrastructure, manifested as disrupted myofibrils and swelling mitochondria. These changes were alleviated by pretreatment with NR1. Taken together, pretreatment with NR1 diminished infarction and tissue injury of the heart induced by I/R.

NRI improves heart function impaired by I/R. As shown in Fig. 3, compared with the sham group, ischemia for 30 min caused a significant decline in LVSP and +dP/dt max and an elevation in −dP/dt max, indicating an impairment of heart function. Reperfusion for 90 min led to a further decline in +dP/dt max as well as a significant decrease in LVSP and a sustained increase in −dP/dt max. Evidently, these impairments were ameliorated by pretreatment with NR1 at the end of reperfusion. However, LVDP did not show any significant difference among groups at each time point during I/R (P > 0.05).

NRI attenuates apoptosis in the myocardium induced by I/R. To determine the antiapoptotic effect of pretreatment with NR1, double staining of cardiac α-actinin and TUNEL in the surrounding infarction areas of the LV myocardium from the various groups was conducted, as shown in Fig. 4. Large numbers of TUNEL-positive cardiomyocytes were observed...
in the I/R group. Noticeably, the number of TUNEL-positive cardiomyocytes in the myocardium of the NR1 pretreatment group was decreased. The statistical result of the percentage of TUNEL-positive cardiomyocytes in surrounding infarction areas is consistent with the qualitative survey, as shown in Fig. 5A.

Apoptosis-related molecules were examined by Western blots, as shown in Fig. 5B. Semiquantitative analysis of the Western blot results is shown in Fig. 5C. As expected, I/R led to a decrease in antiapoptotic Bcl-2 and an increase in proapoptotic Bax and cleaved caspase-3, remarkably. Intriguingly, all alterations were relieved by treatment with NR1. Taken together, these results suggest that pretreatment with NR1 alleviates I/R-induced apoptosis.

NR1 regulates ATP content and ATP synthase subunits impaired by I/R. We explored ATP and AMP in cardiac tissue to address energy metabolism under different conditions. As shown in Fig. 6A, compared with the sham group, I/R had an effect on decreasing ATP levels significantly but did not influence AMP content (P > 0.05), suggesting an impairment of ATP production after I/R. Noticeably, NR1 pretreatment prevented ATP from decline by I/R.

The phosphorylation of AMPK and expression of three subunits of ATP synthase (ATP synthase-α, ATP synthase-β, and ATP5D) in cardiac tissue were determined by Western blot analysis. As shown in Fig. 6B and C, levels of p-AMPK were increased after I/R, and NR1 inhibited this elevation, which agreed with the results of AMP/ATP. For ATP synthase subunits, the expression of ATP5D was significantly reduced after I/R compared with the sham group. Pretreatment with NR1 significantly restrained the decline of ATP5D expression evoked by I/R. There were no notable variations in the expression of ATP synthase-α and ATP synthase-β among the four groups.

NR1 inhibits the expression and activity of ROCK induced by I/R. To determine the involvement of ROCK signaling in I/R injury, the expression of ROCK1 and activity of the ROCK pathway were analyzed by Western blot analysis. As shown in Fig. 7A, levels of ROCK1 were notably upregulated when the myocardium was exposed to I/R compared with the sham group. Impressively, treatment with NR1 significantly reduced the enhancement in the expression of ROCK1. The activity of ROCK was determined by the phosphorylation of MYPT1, the main target protein of ROCK. The results shown in Fig. 7B demonstrate that protein levels of p-MYPT1 were increased under the stimulation of I/R, which were significantly diminished by administration of NR1. These results indicate the potential of NR1 to inhibit the expression and activation of ROCK signaling, which were evoked in the case of I/R injury.

NR1 attenuates OGD/R injury of H9c2 cells. The results of Cell Counting Kit-8 assay showed that at the end of reoxygenation, the viability of H9c2 cells was decreased to approximately half of the control group (Fig. 8A). Treatment with NR1 attenuated the OGD/R-induced depression in cell survival. The released LDH from the cells was tested, as shown in Fig. 8B. Treatment with NR1 markedly decreased the increased release of LDH induced by OGD/R. The results of rhodamine phalloidin staining for the actin skeleton in H9c2 cells are shown in Fig. 8C. Obviously, exposure of H9c2 cells to OGD/R led to a dramatic change in F-actin structure and distribution, with most of the stress fibers localizing in the periphery of the cells.
whereas those in the cytoplasm becoming disintegrated, which was significantly attenuated by treatment with NR1. Ultrastructures of H9c2 cells under different conditions are shown in Fig. 8D. In contrast to normal cells, cells in the OGD/R group showed obvious abnormal structures, with shrunken nuclei, numerous lysosomes, and expanded vacuolated mitochondria. At high magnification (Fig. 8E), most mitochondria in control cells presented as integrated structures and normal size with...
distinct transversely orientated cristae enveloped by an intact outer membrane. However, OGD/R injury resulted in a great number of swollen mitochondria, which displayed spherical structures with disarrayed cristae. Nevertheless, pretreatment with NR1 effectively attenuated the damage of the cell structure, intracellular swelling, and cell organelle abnormality.

To confirm the protective role of ROCK inhibition in OGD/R-induced cell injury, pretreatment with ROCK inhibitor Y-27632 was implemented in H9c2 cells under the disposal of OGD/R as a positive control, and U-46619, a traditional RhoA agonist, was used to activate the RhoA/ROCK pathway. The results revealed that NR1 pretreatment protected H9c2 cells, including improved cell viability, reduced LDH release, and maintenance of the actin skeleton and cell ultrastructure after OGD/R injury, which was similar to the effectiveness of Y-27632. However, U-46619 inhibited the beneficial effect of NR1 on OGD/R-induced injury.

**NR1 ameliorates H9c2 cell apoptosis in OGD/R injury.** Numbers of apoptotic H9c2 cells and the expression of apoptosis-related proteins were determined. Figure 9A shows images of TUNEL staining in H9c2 cells from various groups. Large numbers of TUNEL-positive cells were observed in the OGD/R group. After treatment with NR1 and Y-27632, numbers of TUNEL-positive H9c2 cells decreased, whereas treatment with U-46619 reversed the effect of NR1. The statistical results of the percentage of TUNEL-positive cells (Fig. 9B) were consistent with the qualitative survey.

Bcl-2, Bax, and cleaved caspase-3 were examined by Western blots, as shown in Fig. 9C. As expected, OGD/R led to a decrease in antiapoptotic Bcl-2/Bax and an increase in proapoptotic cleaved caspase-3 (Fig. 9D). However, pretreatment with NR1 inhibited the abnormal expression of these apoptosis molecules, which was reversed by U-46619. On the other hand, when we compared the quantitative results of Bcl-2/Bax between the NR1 group and Y-27632 group, we found that NR1 showed more ability to improve decrease Bcl-2/Bax after OGD/R injury ($P < 0.05$). These results suggest that NR1 could protect cells from OGD/R-induced apoptosis by a mech-

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**Fig. 4. Effect of pretreatment with NR1 on myocardial apoptosis after I/R.** Shown are representative images of TUNEL and cardiac α-actinin double-stained myocardial sections of rats in various groups. Nuclei are stained as blue, cardiac α-actinin is stained as red, and TUNEL-positive cells are green (arrows; a). Bar = 50 μm. n = 6.
anism involving ROCK inhibition, similar to the effect of Y-27632.

NR1 improves ATP synthase activity and upregulates ATP5D in H9c2 cells during OGD/R. The findings in rat heart tissues with respect to energy metabolism were verified in vitro. As shown in Fig. 10A, in H9c2 cells, OGD/R had no effect on AMP levels compared with the control group but dramatically decreased ATP content. Both NR1 and Y-27632 pretreatment elevated the content of ATP after I/R injury but did not influence AMP content. Again, treatment with U-46619...
abated the beneficial role of NR1 in ATP content markedly. As the dominant source of ATP, ATP synthase activity was determined under different conditions. As shown in Fig. 10B, ATP synthase activity in the OGD/R group decreased significantly compared with the control group, and this decrease was blunted by NR1 or Y-27632 treatment. We next determined ATP synthase-α, ATP synthase-β, and ATP5D by Western blot analysis in H9c2 cells in different groups (Fig. 10, C and...
In keeping with previous results in vivo, statistical analysis showed that only the expression of ATP5D decreased significantly after OGD/R compared with the control group. Of note, NR1 or Y-27632 pretreatment restrained the decline of ATP5D expression evoked by OGD/R. Likewise, NR1-elevated ATP5D was downregulated by U-46619. These results point to the potential of NR1 to prevent the decline in ATP production, enhance ATP synthase activity, and regulate ATP5D levels after OGD/R via inhibiting the ROCK pathway. Remarkably, NR1 played a more effective role in enhancing ATP production, improving ATP synthase activity, and upregulating ATP5D expression compared with Y-27632 (P < 0.05).

**NR1 inhibits ROCK1 expression and ROCK pathway activity in H9c2 cell after OGD/R injury.** To manifest the involvement of ROCK signaling in NR1 cardioprotection, the levels of ROCK1 and p-MYPT1 in H9c2 cells were analyzed by Western blot analysis and immunofluorescence. As shown in Fig. 11A, exposure to OGD/R induced increased expression of ROCK1 in H9c2 cells compared with the control group. In contrast, treatment with NR1 significantly reduced the enhancement in the expression of ROCK1, whereas Y-27632, a ROCK inhibitor, did not change the expression of ROCK after OGD/R. The Western blot results shown in Fig. 11B demonstrate that protein levels of p-MYPT1 were increased under the stimulation of OGD/R, which were significantly diminished by the administration of NR1 and Y-27632. Of note, the diminution of p-MYPT1 by NR1 was reversed in the presence of U-46619, a RhoA agonist. These results were verified by immunofluorescent staining (Fig. 11, C and D), where pretreatment with NR1 weakened the increased ROCK1- and p-MYPT1-positive fluorescence in the OGD/R group, Y-27632 changed p-MYPT1 only, and U-46619 significantly enhanced fluorescence of p-MYPT1 after NR1 treatment. The above results indicate the potential of NR1 to inhibit the expression and activation of ROCK signaling.

**DISCUSSION**

The major findings of the present study are as follows. First, pretreatment with NR1 significantly attenuated I/R-induced rat cardiac impairments, as demonstrated by the decreased infarct size and improved myocardium morphology and cardiac function, while cell apoptosis was inhibited and I/R-induced energy metabolism disorder was relieved. Second, evidence from both in vivo and in vitro experiments indicate the potential of NR1 to prevent ROCK signaling from activation induced by I/R.
Finally, we provide evidence showing, for the first time, that compared with classical ROCK inhibitor Y-27632, NR1 could not only alleviate cell injury and apoptosis but also play a larger role in relieving I/R-induced energy metabolism disorder, as shown by the elevation of ATP synthase activity and ATP5D expression by inhibiting the ROCK pathway.

Despite the widespread application of PCI for patients with ischemic heart diseases, the resultant reperfusion injury remains a challenge for clinicians (13). Although reperfusion restores blood flow in the myocardium rapidly, the oxygen and glucose resupply after continuous deprivation induces more serious damage. However, strategies to prevent reperfusion injury and improve PCI outcomes remain limited in the clinic. Panax notoginseng and its derivatives, such as panaxatriol saponins, Rg1, Rb1, and trilinolein, could protect the cardiovascular system by antithrombosis, antiarrhythmia, and antioxidant activity (4, 15, 16). The beneficial effect of NR1 on I/R injury has been demonstrated in the liver, kidney, and gut (5, 17, 20). However, no study has been reported to address the action of NR1 on myocardial injury after I/R, although recently studies (18a, 34a) on some chemicals with similar structures have suggested its potential role in this condition. Our present work demonstrates that pretreatment with NR1 protected the rat heart from I/R-induced structure and function injury, suggesting NR1 as a potential adjuvant therapy for patients presenting with acute myocardial infarction, and pretreatment with NR1 may prevent reperfusion injury and improve PCI outcomes. Although Panax notoginseng is widely used, either alone or in combination with other herbs, in Asian populations, NR1 as a pure chemical has not been applied in the clinic to cope with cardiac I/R injury. Therefore, the dose of NR1 used in the present study had to be determined based on previous studies (5, 17, 20) in animal models on the role of NR1 in I/R injury of the liver, kidney, and gut. Further work is required for clinic translation of the present results.

The heart is among the organs most rich in mitochondria, as profound energy is demanded to support the contraction/relaxation cycle. During ischemia, hypoxia and malnutrition lead to energy metabolism disorder characterized by ATP depletion, resulting in a wide range of detrimental consequences. Poor supply of ATP results in impaired ATP-dependent physiology processes such as F-actin polymerization and filament sliding, which exhibits as imbalanced systolic and diastolic work of the heart (6). Excessive AMP from ATP degradation is the main source of ROS generated from the xanthine oxidase system in the course of reperfusion. ATP depletion leads to dysfunction.
of the Na\textsuperscript{+}-K\textsuperscript{+} pump, which causes a rise in intracellular Na\textsuperscript{+} concentrations, which, in turn, increases Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, thereby raising intracellular Ca\textsuperscript{2+} levels, aggravating Ca\textsuperscript{2+} overloading, free radical generation, and cell swelling (22, 33). Therefore, strategies that are able to improve energy metabolism in the myocardium undergoing I/R challenge would be appealing to limit heart contractile dysfunction and myocardial injury. ATP synthase in the mitochondrion plays a vital role in the energy supply of the cell (34) and is composed of a series of important subunits to perform ATP synthesis. Among these subunits, ATP5D, the gene encoding the \(-\)-subunit, has a critical role in ATP production by polymerizing ATP synthase (14, 25). Consistent with our previous reports (19, 32), the present study revealed a decrease in ATP content and ATP5D expression after cardiac I/R challenge in both in vivo and in vitro experiments. Moreover, no alterations were detected in the expression of ATP synthase-\(-\) and ATP synthase-\(\beta\), two other important subunits composing ATP synthase, in response to I/R. These results suggest that the decrease in ATP5D expression is likely an unique change that occurs after I/R and is responsible for the reduced ATP supply, as previous reports have implied (7, 9, 24). NR1, in addition to inhibiting I/R-induced tissue injury and apoptosis in the heart, was observed to be able to attenuate the decrease in ATP content and ATP5D expression after I/R in the present study, in line with our findings in the rat intestine (17).

ROCK belongs to the AGC (PKA/PKG/PKC) family of serine/threonine kinases and is a major downstream effector of the small GTPase RhoA with multiple functions. A broad spectrum of effects has been shown to be mediated by ROCK in cardiomyocytes, including hypertrophy, apoptosis, and contraction, and a deleterious role of ROCK signaling in myocardial ischemic injury has been previously demonstrated (1, 11, 36). RhoA/ROCK activation was found to aggravate heart I/R injury by mediating myocardial apoptosis and inflammation (37) and inducing cardiac remodeling and fibrosis (18). The mechanisms by which ROCK signaling contributes to I/R injuries include suppressing the reperfusion injury salvage

Fig. 11. NR1 pretreatment inhibits ROCK expression and activation induced by OGD/R in H9c2 cells. A: representative Western blot bands of ROCK1 and semiquantitative analysis of Western blot results of ROCK1 in different groups in H9c2 cells. \(\beta\)-Actin was used as a loading control. Values are means ± SE; \(n = 6\). *\(P < 0.05\) vs. the control group; \#\(P < 0.05\) vs. the OGD/R group; &\(P < 0.05\) vs. the Y-27632 + OGD/R group. B: representative Western blot bands of p-MYPT1 and total MYPT1 and semiquantitative analysis of band intensity of p-MYPT1/MYPT1 in H9c2 cells. Values are means ± SE; \(n = 6\). *\(P < 0.05\) vs. the control group; \#\(P < 0.05\) vs. the OGD/R group; &\(P < 0.05\) vs. the NR1 + OGD/R group. C and D: immunofluorescence staining of ROCK1 (C) and p-MYPT1 (D) in six groups of H9c2 cells. Nuclei were stained with Hoechst 33342 (blue). Arrows indicate overexpressed proteins (red). Bar = 50 \(\mu\)m. \(n = 5\).
kinase pathway, e.g., by phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase signaling (31), increasing the Bax-to-Bcl-2 mRNA ratio and expression of caspase-3 (28), and reassembling myofibers and the cytoskeleton (30). In cardiac I/R injury, the roles of ROCK inhibition by inhibitor Y-27632 that have been reported include reducing infarct size, preventing oxidative stress, lessening inflammation, reducing apoptosis, and enhancing contractile function (10). Particularly, a proteomic study by Cadete et al. (3) showed that Y-27632 results in an increase in LDH and GAPDH, normalization of creatine kinase, and increased levels of two different molecular fragments of ATP synthase-α during cardiac I/R injury, suggesting the involvement of ROCK in the impaired energy production. In support of this notion, our study provides direct evidence showing, for the first time, a significant increase in ATP content, ATP synthase activity, and ATP5D expression by Y-27632 pretreatment in I/R-challenged H9c2 cells. These results confirm that ROCK signaling is a modulator of ATP5D expression and ATP production. Nonetheless, the details of this signaling pathway await further study.

Despite the potential clinical importance of ROCK inhibition, there are few ROCK inhibitors approved for human use (30). Evaluation of more selective ROCK inhibitors will help to validate ROCK as a crucial target in myocardial ischemic diseases. In our research, we found that NR1, a main constituent of Panax notoginseng, significantly inhibited ROCK1 expression and activation both in vitro in H9c2 cells exposed to OGD/R and in vivo after I/R injury in the rat heart, suggesting that it may act as a new ROCK inhibitor in heart protection. When we compared the effects of Y-27632 and NR1, we found that NR1 improved cell viability, maintained normal actin cytoskeleton organization and distribution, protected cell and organelle morphology, and inhibited cell apoptosis, much like Y-27632. Remarkably, in the matter of energy metabolism and ATP production, NR1 displayed a more beneficial role than Y-27632.

Nonetheless, the present study has left some questions open. First, our experiments showed no change in the expression of Y-27632. Remarkably, in the matter of energy metabolism and ATP production, NR1 displayed a more beneficial role than Y-27632. In conclusion, NR1 pretreatment attenuated myocardium injury and cardiac malfunction after I/R. Particularly, NR1 treatment inhibited the expression and activity of ROCK and elevated ATP5D expression and ATP content, suggesting that ROCK signaling mediates ATP5D expression and contributes to the role of NR1 in attenuating ATP metabolism disorder. The present study provides evidence supporting the application of NR1 as a management therapy in the clinic for patients undergoing cardiac I/R challenge and opens the door for the development of new medications that target ROCK to improve I/R-induced cardiac energy deficiency.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


