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Fasudil Hydrochloride Hydrate, a Rho-Kinase Inhibitor, Suppresses Isoproterenol-Induced Heart Failure in Rats via JNK and ERK1/2 Pathways

Na Wang,[1] Peng Guan,[2] Jian-Ping Zhang,[1] Ya-Qing Li,[2] Yan-Zhong Chang,[2] Zhen-Hua Shi,[2] Feng-Yun Wang,[1] and Li Chu[1*]

[1] *Department of Pharmacology, School of Basic Medicine, Heibei Medical University, Shijiazhuang 050091, Hebei, China*

[2] *The Key Laboratory of Animal Physiology, Biochemistry and Molecular Biology of Hebei Province, Hebei Normal University, Shijiazhuang 050016, Hebei, China*

ABSTRACT

The Rho-kinase (ROCK) plays an important role in the pathogenesis of heart injury. Recent cellular and molecular biology studies indicated a pivotal role of the RhoA/ROCK cascade in many aspects of cardiovascular function such as heart failure, cardiac hypertrophy, and ventricular remodeling following myocardial infarction. However, the signal transduction of RhoA/ROCK and its down-stream signaling pathways remains elusive, and the mechanism of ROCK-mediated isoproterenol (ISO)-induced heart failure is still not thoroughly understood. In the present study, we investigated the effect of the ROCK inhibitor, fasudil hydrochloride hydrate, on ISO-induced heart failure and the potential relationship of RhoA/ROCK to the extracellular signal-regulated kinases (ERK) and the c-jun NH 2-terminal kinase (JNK) pathways. Male Sprague-Dawley (SD) rats, maintained on a normal diet, were randomly divided into four groups given control, ISO alone, ISO with low-dose fasudil, or ISO with high-dose fasudil treatments. Fasudil effectively inhibited ISO-induced heart failure, as evaluated by biometric, hemodynamic, and histological examinations. Consistently, ISO-induced ROCK-1 mRNA expression and myosin phosphatase target subunit-1 (MYPT-1) phosphorylation were markedly suppressed by fasudil. In addition, fasudil significantly decreased ISO-induced JNK activation, ERK translocation to the nucleus and subsequent c-fos, c-jun expression and upregulated c-FLIP$_L$ expression. Taken together, these results indicate that the RhoA/ROCK pathway is essential for ISO induced heart failure, which can be effectively suppressed by fasudil. J. Cell. Biochem. 112: 1920–1929, 2011.

KEY WORDS: FASUDIL; HEART FAILURE; ISOPROTERENOL; RHO-KINASE

Isoproterenol (ISO) is a potent agonist of β-adrenoceptors. ISO-induced heart failure is widely used as an experimental animal model. As the pathophysiological changes following ISO administration are comparable to those that take place in humans, the model is reliable and reproducible, and the associated effects of hypertrophy, apoptosis, fibrosis, arrhythmias, myocyte loss, and fibrosis with progression to heart failure are well-characterized [Szabo et al., 1975].

Rho kinase (ROCK), the first Rho effector to be described, is a serine/threonine kinase that is important in fundamental processes of cell migration, cell proliferation and cell survival. There are two isoforms of ROCK, known as ROCK I and II. ROCK I shows the highest expression level in non-neuronal tissues, whereas ROCK II is preferentially expressed in the brain. The knowledge of the involvement of ROCKs in the cardiovascular system came mostly from studies utilizing pharmacological inhibitors. Fasudil, a ROCK inhibitor, was shown to decrease ischemia–reperfusion injury, infarct size and myocardial fibrosis in response to experimental myocardial infarction and in a rat model of chronic hypertension induced congestive heart failure [Satoh et al., 2003].

The extracellular signal-regulated kinases (ERK) signaling pathway has been implicated in cardiac pathology, such as the progression from cardiac hypertrophy to failure [Munzel et al., 2005]. For example, samples obtained from patients with end-stage

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*Correspondence to: Prof. Li Chu, PhD, Department of Basic Courses, Heibei Medical University, Shijiazhuang 050091, Hebei Province, China. E-mail: chuli0614@126.com

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heart failure due to dilated cardiomyopathy revealed a marked increase in ERK1/2 activity [Takeishi et al., 2002]. Transgenic mice in which ERK1/2 was activated by cardiac overexpression of either Ras or MAP kinase kinase 1 (MEK1) developed massive cardiac hypertrophy [Bueno et al., 2000; Zheng et al., 2004]. The ERK pathway also has been linked to cardiac hypertrophy and contractile dysfunction induced by ISO [Munzel et al., 2005]. Prasanna et al. showed that cardiac myocyte apoptosis in ß1 integrin-deficient mice is induced via the involvement of the the c-jun NH 2-terminal kinase (JNK)-dependent mitochondrial pathway [Krishnamurthy et al., 2007]. Previous studies showed that multiple signaling pathways such as the ERK1/2 and JNK pathways are involved in ISO-induced myocardial hypertrophy and heart failure [Zhang et al., 2005; Zhang et al., 2010]. Moreover, the RhoA/ROCK pathway provides promising pharmacological targets for treatment of a variety of cardiovascular diseases. However, there has been limited study of the relationship between the RhoA/ROCK and MAPK signaling pathways.

Based on the information above, we hypothesized that RhoA/ROCK activation is important in mediating ISO induced heart failure and that specific crosstalk exists between the ROCK and MAPK signaling pathways during this process. We introduced several lines of evidence indicating that activation of RhoA/ROCK is specifically required for ISO-induced JNK activation, ERK translocation to the nucleus and subsequent heart failure and that fasudil is effective for reversing the disease. These data support a novel role of the RhoA/ROCK cascade in myocardium remodeling and further provide insight into the molecular mechanisms underlying the beneficial effects of fasudil against heart failure.

MATERIALS AND METHODS

ANIMAL PREPARATION

Adult male Sprague-Dawley rats (weight 210 ± 10 g) were purchased from the Laboratory Animal Center (Hebei Medical University, China). Rats were kept under a 12-h light/dark cycle (lights on 8:00 am) in a temperature- and humidity-regulated facility. Animals were allowed to access food and water *ad libitum*. All protocols for rats were in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and were approved by the animal Ethics and Use Committee of Hebei Science and Technical Bureau in the People's Republic of China. All efforts were made to reduce the number of animals used and their suffering.

ADMINISTRATION OF CHEMICALS AND DRUGS

All rats were randomly assigned to control (CONT), ISO alone (ISO), low-dose fasudil treatment plus ISO (FAS_L + ISO) or high-dose fasudil treatment plus ISO (FAS_H + ISO) groups. ISO (Hefeng Co., Shanghai, China) was administered once daily by intraperitoneal injection at 5 mg/kg/day for 1 week. Control animals received an injection of the equivalent volume of saline. Fasudil (Hongri Co., Tianjin, China, 2, 10 mg/kg/day) was injected subcutaneously in the FAS_L + ISO group and FAS_H + ISO group. The rats given ISO administration were all alive after 1 week. All evaluations were performed 24 h after the last ISO administration.

HISTOLOGICAL ANALYSIS

The body weights and cardiac weights were collected. The left ventricle (LV) was fixed with 10% paraformaldehyde in PBS for 2 days at 4°C, embedded in paraffin and cut into 5 μm slices, which were stained with hematoxylin-eosin (HE) for morphological analysis and with Masson's stain for fibrosis analysis. Photographs of six LV sections from the CONT ($n = 3$), ISO ($n = 3$), FAS_L + ISO ($n = 3$) and FAS_H + ISO ($n = 3$) groups were taken at 400× magnification, and cross-sectional images of cardiac myocytes were digitized using a digital microscope (ZEISS Digital Camera, German).

ACQUISITION OF HEMODYNAMIC PARAMETERS

Twenty-four hours after the last injection, animals were anesthetized with tribromoethanol (15 μl of 2.5% solution per g of body weight), which has been demonstrated to have a less depressive effect on cardiac function than with other commonly used anesthetics. A miniature pressure transducer (Chengdu instrument Co., Chengdu, China) was inserted into the LV via the right carotid artery. The heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVDP), and the first derivatives of left intraventricular pressure (rate of pressure development + dP/dt and rate of pressure decrease − dP/dt) were monitored continuously, recorded and analyzed after 10 min of stabilization by using the MS4000U-1C Quantitative recording of biological signals analysis system (the Guangzhou Science and Technology Co., Ltd. Feilong up production, Guangzhou, China).

ELECTROCARDIOGRAPHY

To detect heart failure, 12-lead electrocardiography was performed in rats anesthetized with tribromoethanol. Body surface electrocardiograms (ECGs) were obtained with rats in the supine position. ECGs were recorded using three needle electrodes placed subcutaneously: red on left lower extremity, green on right upper extremity and black on right lower extremity. The distal portions of the leads were secured in positions that approximated those of the lead II of a standard ECG. All rats breathed spontaneously throughout this procedure. We chose to perform echocardiography with a straightforward, easily reproducible approach by capturing the whole cardiac cycle using a RM6240C Multi-channel physiological signal acquisition and processing system (Chengdu instrument Co., Chengdu, China).

WESTERN BLOTTING

For Western blotting analysis, tissue samples from the LV of rats were frozen in liquid nitrogen and stored at −70°C until further processing. Proteins were extracted from fresh-frozen myocardium. The tissues were homogenized and lysed with RIPA lysis buffer containing 100 mg/ml PMSF, 1 mg/ml aprotinin. The lysate was collected, kept on ice for 15 min and centrifuged at 12,000 × *g* at 4°C for 10 min. The pellet (including the nuclei) was used to detect p-ERK1/2. Briefly, equal amounts of protein (30–50 μg) were loaded and separated on a 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane (Millipore, USA). After blocking with non-fat milk, the membrane was incubated with polyclonal rabbit anti-rat myosin phosphatase target subunit-1 (MYPT-1), p-MYPT1,

TABLE I. Primers Used in This Study

Primer name	Accession number[a]	Primers	Predicted product size (bp)
ROCK I	NM_031098	GAGCAACTACGATGTGCCTGAAAAGT GATGACGTTTGATTTCCTCTAC	512
c-fos	NM_010234	AGAATCCGAAGGGAAAGGAA CTTCTCCTTCAGCAGGTTGG	150
c-jun	NM_010591	CCTTCTACGACGATGCCCTCAA GGGGTCGGTGTAGTGGTGATGT	259
c-FLIP$_L$	NM_009805	GTCTGCTGAAGTCATCCATCAG CTTATGTGTAGGAGAGGATAAG	230
GAPDH	NM_017008	GTCTACTGGCGTCTTCA GGGTAGGAACACGGAAG	506

[a]GenBank accession numbers (http://www.ncbi.nlm.nih.gov).

ERK1/2, p-ERK1/2, JNK, p-JNK, c-FLIP$_L$ or GAPDH antibody (Santa Cruz Biotechnology, USA, diluted at 1:1000). Specific proteins were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (Zhongshan goldenbridge, China) and visualized by an ECL system (Fuji, Japan).

RT-PCR

Expressions of ROCK I, c-fos, c-jun, c-FLIP$_L$ and GAPDH in cardiomyocytes were examined by semi-quantitative RT-PCR. Total RNA was extracted from fresh-frozen myocardium using the Trizol Reagent (USA, Invitrogen). cDNA was synthesized according to the manufacturer's instructions of the Reverse Transcription kit (TaKaRa, Japan) and then amplified with a Multiplex polymerase chain reaction (PCR) kit (TaKaRa, Japan) using specific primers. The sequences of the oligonucleotides are summarized in Table I.

DATA ANALYSIS

Data are presented as mean ± S.E.M. Differences between the mean values of the densitometric readings were tested by one-way analysis of variance (ANOVA) for repeated measurements and the Bonferroni multiple-range test. A probability value of $P < 0.05$ was considered to be statistically significant.

RESULTS

COLLECTION OF BIOMETRIC PARAMETERS AND HISTOLOGICAL ANALYSIS

Four groups of rats (CONT, ISO, FAS$_L$ + ISO and FAS$_H$ + ISO) were treated for 1 week as described in the Materials and methods. Overall mortality in rats 1 week after receiving ISO administration was 0%. Biometric parameters collected on the rats are presented in Table II. Heart weight (HW) to-body weight (BW) ratio (HW/BW) was increased by about 41.53% in ISO group as compared to CONT group (ISO: 4.26 ± 0.14 versus CONT: 3.01 ± 0.04, $P < 0.01$). Moreover, LV wet weight (WW) /BW ratio (LV WW/BW) was increased to 127.60% (ISO: 3.19 ± 0.06 versus CONT: 2.50 ± 0.28, $P < 0.05$) and LV dry weight DW/BW ratio (LV DW/BW) was increased to 138.18% (ISO: 0.76 ± 0.01 versus CONT: 0.55 ± 0.07, $P < 0.05$). After being administrated with fasudil, HW/BW was significantly reduced in FAS$_L$ + ISO group (FAS$_L$ + ISO: 3.77 ± 0.10 versus ISO: 4.26 ± 0.14, $P < 0.05$) and FAS$_H$ + ISO group (FAS$_H$ + ISO: 3.44 ± 0.08 versus ISO: 4.26 ± 0.14, $P < 0.01$). WW/BW of LV was significantly reduced by 15.05% in FAS$_L$ group (FAS$_L$ + ISO: 2.71 ± 0.16 versus ISO: 3.19 ± 0.06, $P < 0.05$) and 28.84% in FAS$_H$ group (FAS$_H$ + ISO: 2.27 ± 0.02 versus ISO: 3.19 ± 0.06, $P < 0.01$) as compared to ISO group. DW/BW of LV showed a similar tendency as LV WW/BW (FAS$_L$ + ISO: 0.63 ± 0.06 versus ISO: 0.76 ± 0.01, $P < 0.05$; FAS$_H$ + ISO: 0.60 ± 0. 01 versus ISO: 0.76 ± 0.01, $P < 0.01$).

As shown in Figure 1A, the LV were hypertrophied significantly in the ISO group, but could be reversed by fasudil treatment. As shown in Figure 1B and C, HE was for morphological analysis and Masson's stain was for fibrosis analysis. Treatment with isoproterenol resulted in marked myocyte loss and increased fibrosis, primarily limited to the subendocardium of the LV free wall and septum, not extending to the epicardium. The collagen fraction was determined by measuring the area of blue-stained tissue within a given field with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The stained area was calculated as a percentage of the total area within an image. The blue-appearing collagen of the LV endocardium was significantly higher in isoproterenol-treated rats (about 84.56%). However, the progressive LV fibrosis was attenuated by fasudil and the effect was in a dose dependent manner (about 43.79% in FAS$_L$ + ISO and 20.01% in FAS$_H$ + ISO).

HEMODYNAMIC PARAMETERS AND ELECTROCARDIOGRAPHY

Treatments of rats with a single dose of ISO (5 mg/kg/day) caused significant increases ($P < 0.01$) in HR and left ventricular end-diastolic pressure (LVEDP) compared to the control value, indicating

TABLE II. Comparison of Cardiac Wet Weights and Dry Weights Among the Four Groups

Group	BW (g)	HW (mg)	LV WW (mg)	LV DW (mg)	HW/BW (mg/g)	LV WW/BW (mg/g)	LV DW/BW (mg/g)
CONT	251 ± 8	756 ± 14	626 ± 56	138 ± 16	3.01 ± 0.04	2.50 ± 0.28	0.55 ± 0.07
ISO	195 ± 9	828 ± 35	620 ± 28	148 ± 7	4.26 ± 0.14**	3.19 ± 0.06*	0.76 ± 0.01*
FAS$_L$ + ISO	206 ± 7	776 ± 17	559 ± 40	130 ± 14	3.77 ± 0.10#	2.71 ± 0.16#	0.63 ± 0.06#
FAS$_H$ + ISO	229 ± 4	787 ± 16	519 ± 5	138 ± 4	3.44 ± 0.08##	2.27 ± 0.02##	0.60 ± 0. 01##

BW, body weight; HW, heart weight; LV, left ventricular; HW/BW, Heart weight (HW) to-body weight (BW) ratio; LV WW/BW, LV wet weight (WW)-to-body weight (BW) ratio; LV DW/BW, LV dry weight (DW)/BW ratio. *$P < 0.05$, **$P < 0.01$, vs. CONT group; #$P < 0.05$, ##$P < 0.01$, vs. ISO group.

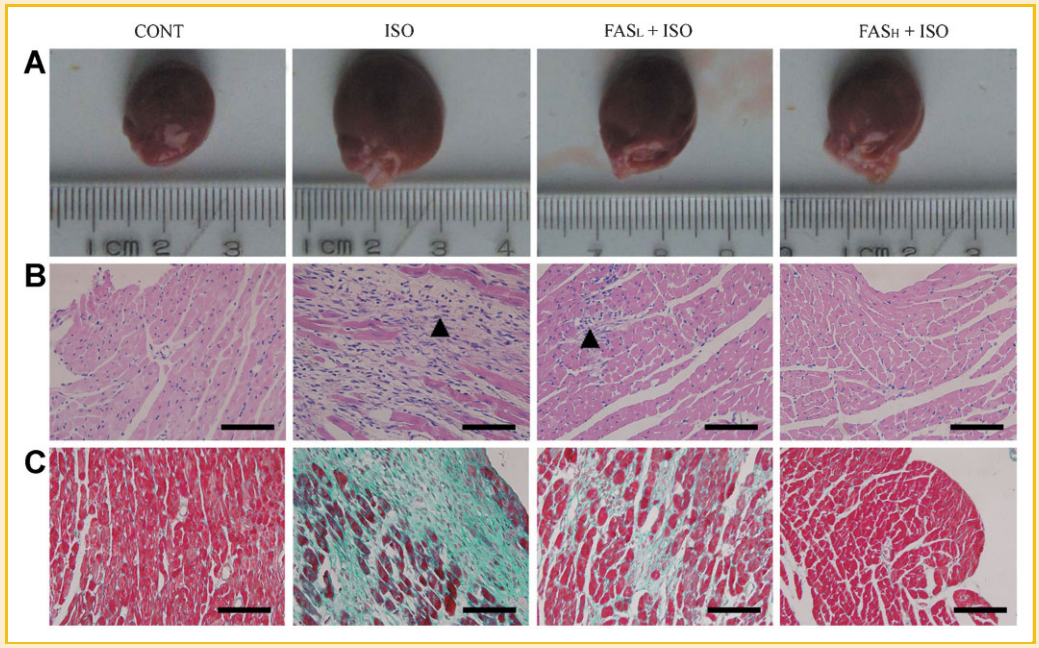

Fig. 1. Histology of rat hearts in the four treatment groups. A: Representative hearts of rats in each of the four indicated groups. B: HE staining. C: Masson's staining. The cross-sectional views of the rat hearts in the four groups. Notice the increased fibrosis in the ISO (B: black arrow, C: blue stain) with almost complete fibrosis at the endocardium (Endo) and diffuse fibrosis of the posterior left ventricle (LV). Fibrosis in the $FAS_L + ISO$ and $FAS_H + ISO$ groups (black arrow) is ameliorated by fasudil. Moreover, the decrease in fibrosis was dose-related in the $FAS_L + ISO$ and $FAS_H + ISO$ groups. Bar = 10 μm.

LV haemodynamic overload. On the other hand, ISO caused significant decreases ($P < 0.01$) in left ventricular systolic pressure (LVSP) and $\pm dp/dt_{max}$ compared to the control values. LVSP and $\pm dp/dt_{max}$ are sensitive to changes in preload and afterload. Haemodynamic parameters changes indicated left ventricular dysfunction in ISO rats. Treatment of ISO-injected rats with fasudil for 1 week markedly normalized the heart function. Both systolic and diastolic function were significantly enhanced, and LVSP, $\pm dp/dt_{max}$ and LVEDP were all markedly improved in the $FAS_L + ISO$ group ($P < 0.05$) and $FAS_H + ISO$ group ($P < 0.01$) compared with the ISO group (Table III). Figure 2 shows the standard ECGs traces of lead II from the CONT rats during baseline (A), ST segment depression with a negative T-wave (B) and typical arrhythmias in the ISO group (C, D, E). Treatment of ISO-injected rats with fasudil for 1 week ameliorated the heart dysfunction (F, G).

EFFECT OF FASUDIL ON ISO-MEDIATED ROCK ACTIVATION

As shown in Figure 3A, total RNA was extracted from the LVs of the CONT, ISO, $FAS_L + ISO$ and $FAS_H + ISO$ groups. ISO caused a significant up-regulation of ROCK I mRNA expression compared with the CONT group, and treatment with fasudil resulted in a significant reduction in ROCK I mRNA expression (Fig. 3B, $P < 0.01$). Because MYPT-1 is a major downstream target of RhoA/ROCK-mediated Ca^{2+} sensitization and a regulator of myosin light chain (MLC) activation. We tested the activation of RhoA/ROCK pathway by the amount of phosphorylated MYPT-1. As shown in Figure 3C, ISO caused phosphorylation of MYPT-1, which was inhibited by fasudil in a dose-dependent manner ($P < 0.01$).

FASUDIL REDUCES ISO-INDUCED ACTIVATION OF ERK

The Ras/Raf/MEK/ERK signal transduction pathway plays an important role in the regulation of myocardial hypertrophy and heart failure in response to extracellular signals [Wang et al., 2005; Oudit et al., 2008]. On this basis, we determined the effect of fasudil on the phosphorylation status of ERK in the nucleus as it may participate in ISO-induced heart failure. As shown in Figure 4, ISO increased nuclear translocation of ERK as determined by Western blot analysis, while fasudil reduced this ISO-induced p-ERK to various degrees ($P < 0.01$).

TABLE III. Hemodynamic Assessments in the Four Groups

Group	HR (bpm)	LVSP (mmHg)	LVEDP (mmHg)	$+dp/dt_{max}$ (mmHg/s)	$-dp/dt_{max}$ (mmHg/s)
CONT	343 ± 9.6	143 ± 5.8	$4.77 + 0.38$	8311 ± 437	7116 ± 272
ISO	$424 \pm 8.2^{**}$	$98 \pm 8.2^{**}$	$15.37 + 0.49^{**}$	$4339 \pm 232^{**}$	$3623 \pm 598^{**}$
$FAS_L + ISO$	$372 \pm 7.3^{\#\#}$	$126 \pm 6.7^{\#}$	$11.73 + 1.31^{\#}$	$5787 \pm 246^{\#\#}$	$5130 \pm 1344^{\#}$
$FAS_H + ISO$	$362 \pm 6.9^{\#\#}$	$140 \pm 8.0^{\#}$	$7.60 + 0.33^{\#\#}$	$7861 \pm 324^{\#\#}$	$6660 \pm 334^{\#\#}$

Values are mean ± S.E.M. $^{**}P < 0.01$, vs. CONT group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. ISO group, n = 6.

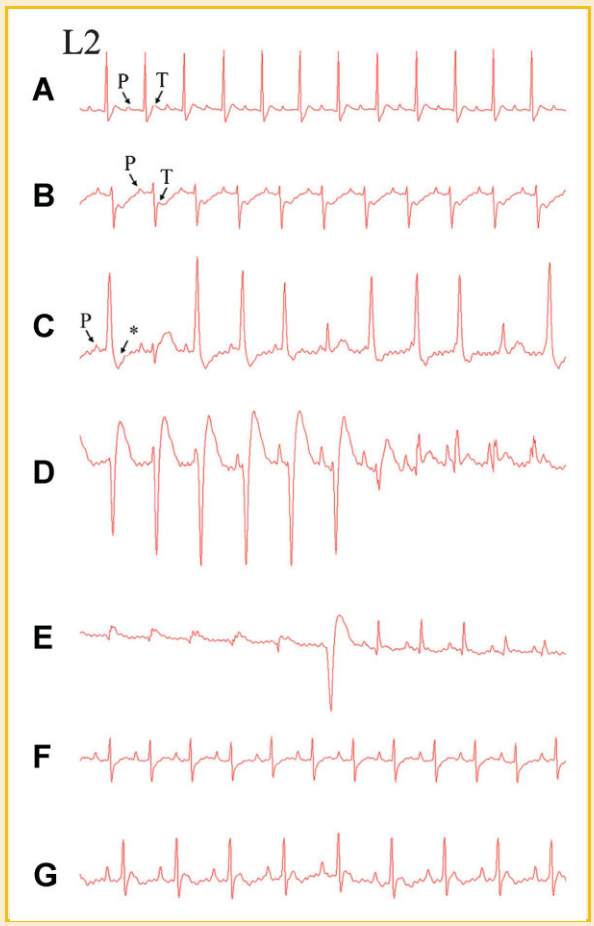

Fig. 2. Representative ECG recordings from the CONT, ISO, FAS_L + ISO, and FAS_H + ISO groups. The asterisk indicates a ST segment depression with a negative T-wave. Similar results were observed in at least 8/12 rats. A: A standard ECG trace from a CONT rat during baseline; B: ST segment depression with a negative T-wave in a rat heart fromthe ISO group; C, D, and E: Typical arrhythmias in a rat heart from the ISO group; F and G: Treatment of ISO-injected rats with fasudil for 1 week ameliorated the heart dysfunction.

FASUDIL ATTENUATES ISO-INDUCED JNK ACTIVATION

To further investigate the crosstalk between the RhoA/ROCK and MAPK signaling pathways, we observed the effect of fasudil on ISO-induced JNK activation. As shown in Figure 5, ISO increased the activities of JNK phosphorylation to 189.24% ($P < 0.01$). Compared with ISO group, fasudil reduced JNK phosphorylation by about 24.71% ($P < 0.01$) at low dose and 43.37% ($P < 0.01$) at high dose. These results suggested the presence of signaling crosstalk between RhoA/ROCK and JNK in ISO-induced heart failure.

EFFECT OF FASUDIL ON C-FOS AND C-JUN mRNA EXPRESSION

Since c-fos binds to c-jun to form activator protein-1 (AP-1) that plays an important role in the MAPK signaling pathway, we measured the mRNA expression of c-fos and c-jun in the LVs of the different groups as described in Materials and methods. As shown in Figure 6, when the rats were administrated by ISO, the expression of c-fos and c-jun mRNA quickly increased, reaching about 252.92% ($P < 0.01$) and 247.45% ($P < 0.01$), respectively. The increases of c-fos and c-jun mRNA expression by ISO were inhibited by high-dose fasudil by approximately 30.84% ($P < 0.01$) and 41.25% ($P < 0.01$), respectively. There were no significant decreases in the c-fos and c-jun mRNA expression in low-dose fasudil administrated rats.

EFFECT OF FASUDIL ON c-FLIPL mRNA AND PROTEIN EXPRESSION

$c\text{-}FLIP_L$ is widely believed to be an antagonist to the death receptor-initiated apoptotic pathways. A recent report also suggests that c-FLIP may protect against certain types of myocyte death, and c-FLIP is of special interest in the heart because of its possible role in preventing apoptosis [Imanishi et al., 2000]. AP-1 binds to the $c\text{-}FLIP_L$ promoter, represses its transcriptional activity and reduces $c\text{-}FLIP_L$ mRNA and protein levels. Therefore, we determined both the mRNA and protein expressions of $c\text{-}FLIP_L$ [Li et al., 2007; Zhang et al., 2007]. As shown in Figure 7, the $c\text{-}FLIP_L$ mRNA expression decreased in the ISO group. By contrast, treatment with fasudil resulted in a significant increase ($P < 0.01$). Simultaneously, the $c\text{-}FLIP_L$ protein levels changed similarly to that of the $c\text{-}FLIP_L$ mRNA.

DISCUSSION

ISO-induced heart failure is a reliable, reproducible and well-characterized model of cardiac hypertrophy associated with apoptosis, fibrosis, arrhythmias, myocyte loss and myocardial ischemia [Szabo et al., 1975]. The RhoA/ROCK pathway has been implicated in diverse cardiovascular diseases such as cardiac hypertrophy and heart failure, and it may also be substantially involved in ISO-induced heart failure [Yatani et al., 2005]. However, the mechanisms of action of ROCK inhibitors leading to beneficial effects in ISO-induced heart failure are largely unknown. The signals transduced by Rho/ROCK activation and its downstream signaling effects that mediate heart failure are not fully understood. Fasudil, a novel ROCK inhibitor, has been used clinically for ameliorating pacing-induced myocardial ischemia in patients with effort angina [Otsuka et al., 2008]. Moreover, fasudil improves increased vascular resistance and impaired vasodilation of the forearm in patients with heart failure [Kishi et al., 2005]. Because of the associations of ROCK and cardiomyocyte remodeling, we undertook this study to evaluate the influence of fasudil on ISO-induced cardiomyocyte hypertrophy in SD rats and to examine the mechanism of action. The biometric data demonstrated that fasudil markedly suppressed ISO-induced cardiac hypertrophy. Meanwhile, hemodynamic parameters were also reduced by fasudil treatment. These results were in line with the results of the histology assay and ECG, together indicating that fasudil effectively decreased the cardiac hypertrophy, fibrosis and arrhythmias of heart failure.

Rho A is one of the effectors of the small GTP-binding protein Rho. It cycles between a GDP-bound inactive state and a GTP-bound active state [Bar-Sagi and Hall, 2000; Katoh et al., 2001]. ROCK is the best-known downstream effector of Rho A. The RhoA-GTP activates ROCKs, which in turn phosphorylates a non-catalytic subunit of MYPT-1 at Thr 696 to inhibit the myosin phosphatase activity

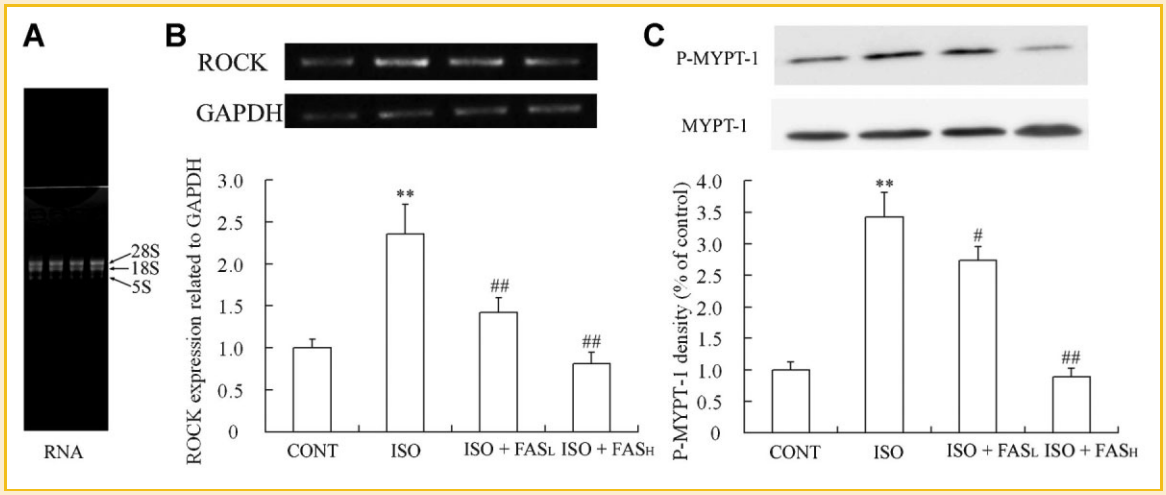

Fig. 3. Effect of fasudil on ROCK I mRNA expression in the LVs. A: Total RNA was prepared from rat LV of CONT, ISO, FAS_L + ISO and FAS_H + ISO groups and analyzed by RT-PCR. The product (512 bp for rat ROCK I) was examined by agarose gel electrophoresis and stained with ethidium bromide. B: The mRNA level of GAPDH (506 bp) was also determined as a control. The level of ROCK I mRNA expression is presented as mean ± S.E.M. (C) Effect of fasudil on ISO-induced ROCK activation as measured by myosin phosphorylation of MYPT-1 (p-MYPT-1 Thr^{696}). Lysates of the LV from the CONT, ISO, FAS_L + ISO and FAS_H + ISO groups were analyzed by immunoblotting to detect p-MYPT-1 or total MYPT-1. The extent of MYPT-1 phosphorylation was quantified by densitometric measurements of the p-MYPT-1/MYPT-1. Data are shown as the mean ± S.E.M. $^{**}P < 0.01$, vs. CONT group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. ISO group, n = 6.

[Ohama et al., 2003]. Therefore, the effect of fasudil on ISO-induced MYPT-1 phosphorylation was examined. The results suggested a significant ISO-induced up-regulation of ROCK-1 mRNA expression and a rapid phosphorylation of MYPT-1 at Thr^{696} in the heart. Treatment of the heart with fasudil significantly inhibited the ROCK-1 mRNA expression and MYPT-1 phosphorylation induced by ISO. These results indicate that ISO led to ROCK-dependent Thr^{696} phosphorylation of MYPT-1, which represents a selective marker of the ROCK activation in the heart.

ISO-mediated ERK activation has been demonstrated to play an important role in cell hypertrophy and proliferation in various cardiac cells, including neonatal and adult rat ventricular myocytes [Yamazaki et al., 1997; Zou et al., 1999] and cardiac fibrosis [Murasawa et al., 1998; Leicht et al., 2000]. Jihee et al. found that ISO

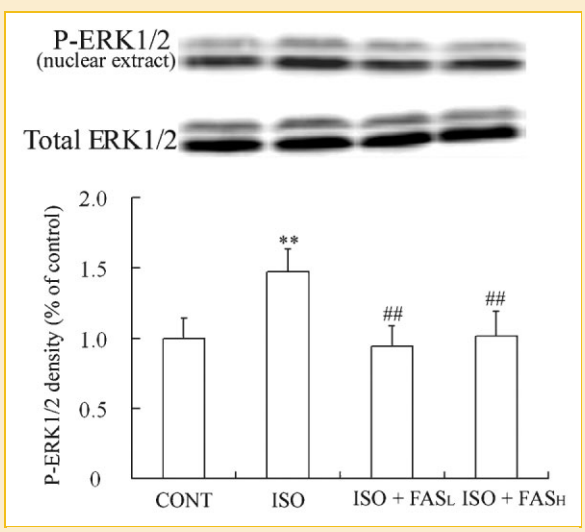

Fig. 4. Effect of fasudil on ISO-induced translocation of p-ERK1/2 into the nucleus. Fasudil reduced ISO-induced nuclear p-ERK1/2 levels. The levels of p-ERK1/2 in the nucleus were determined by Western blotting analysis of the cellular nuclear extracts using the p-ERK antibody. Data are shown as the mean ± S.E.M. $^{**}P < 0.01$, vs. CONT group; $^{\#\#}P < 0.01$, vs. ISO group, n = 6.

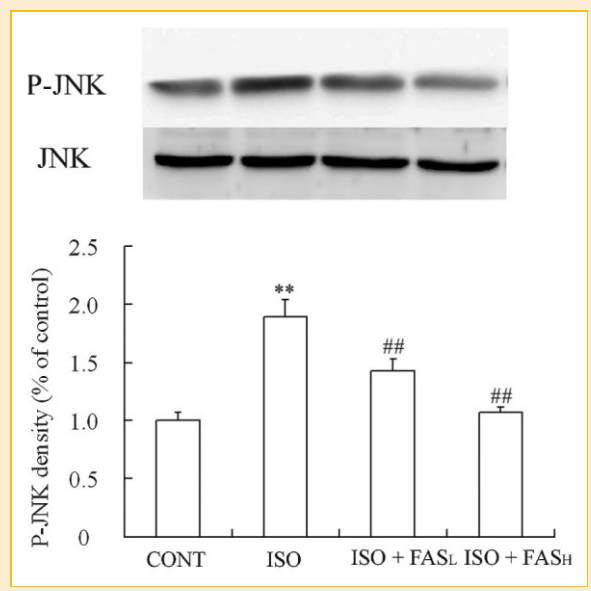

Fig. 5. Effect of fasudil on ISO-induced JNK activation. Fasudil reduced ISO-induced p-JNK elevation. Lysates were probed with antibodies against p-JNK or total JNK. The extent of JNK phosphorylation was quantified by the density of p-JNK/JNK. Data are shown as the mean ± S.E.M. $^{**}P < 0.01$, vs. CONT group; $^{\#\#}P < 0.01$, vs. ISO group, n = 6.

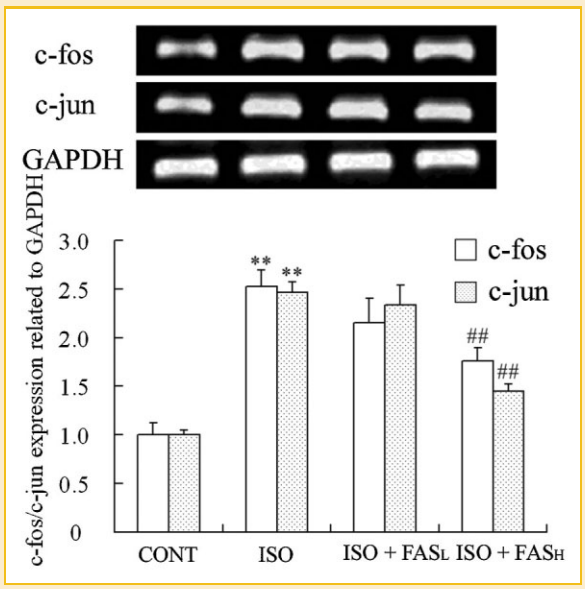

Fig. 6. Effect of fasudil on the c-fos and c-jun mRNA expression in rat LV of CONT, ISO, FAS_L + ISO and FAS_H + ISO groups. Fasudil reduced ISO-induced c-fos and c-jun mRNA expression. After total RNA extraction, RT-PCR was performed as described in Materials and methods. Intensity of c-fos and c-Jun were standardized to that of GAPDH. Data are presented as mean ± S.E.M. $^{**}P < 0.01$, vs. CONT group; $^{\#\#}P < 0.01$, vs. ISO group, n = 6.

stimulates DNA synthesis via ERK in adult rat cardiac fibroblasts [Kim et al., 2002]. However, Colombo et al. demonstrated that ISO-mediated stimulation of protein synthesis does not require ERK activity in neonatal rat cardiac fibroblasts [Colombo et al., 2001]. In view of this controversy and the paucity of data available on ERK activity stimulated by ISO in cardiomyocytes, the present study was designed to investigate the effect of fasudil on nuclear translocation of ERK induced by ISO in the adult rat heart. The phosphorylation of ERK was significantly increased in the ISO group, while fasudil inhibited ERK phosphorylation in the nucleus. Activation of ERK occurs in the cytoplasm, but to exert many of its actions ERK must translocate into the nucleus. Recently, it has been reported that translocation of ERK to the nucleus is dependent on ROCK for any mitogenic event, and the activation of transcription factor Elk-1 depends on both ROCK and ERK, providing supportive evidence that translocation of ERK to the nucleus requires ROCK activation. This is consistent with our result that ERK translocates to the nucleus by ROCK activity, which may account for the fact that fasudil inhibits ROCK-1 mRNA expression. Certainly, further studies are needed to investigate the mechanism.

Previous studies showed that multiple signaling pathways such as ERK1/2 and JNK pathways are involved in ISO-induced myocardial hypertrophy and heart failure [Zhang et al., 2010]. Therefore, we investigated the possible signaling crosstalk between ROCK and the JNK activation induced by ISO in this study. As shown in our results, ISO caused the increase of JNK phosphorylation, which was significantly reduced by fasudil. Activation of JNK signal transduction cascades has been implicated in the regulation of hypertrophic and apoptotic responses in the myocardium [Liang and Molkentin, 2003]. Remondino et al. observed a greater increase in JNK activation in ß1 integrin-deficient mice after infusion with ISO, and activation of JNK was suggested to play a pro-apoptotic role in ISO-stimulated apoptosis [Remondino et al., 2003]. By contrast, we found evidence of crosstalk between the RhoA/ROCK and JNK signaling pathways in mediating the ISO-induced myocardial hypertrophy and apoptosis. Thus, we can conclude that fasudil inhibits ISO-induced myocardial hypertrophy and apoptosis partially by blocking JNK activation.

Activation of ERK is responsible for downstream actions that enhance expression of genes important in cell cycling, such as c-fos and c-jun [Murphy et al., 2002; Oldenhof et al., 2002]. Moreover, JNK activation induces expression of c-fos and c-jun [Karin et al.,

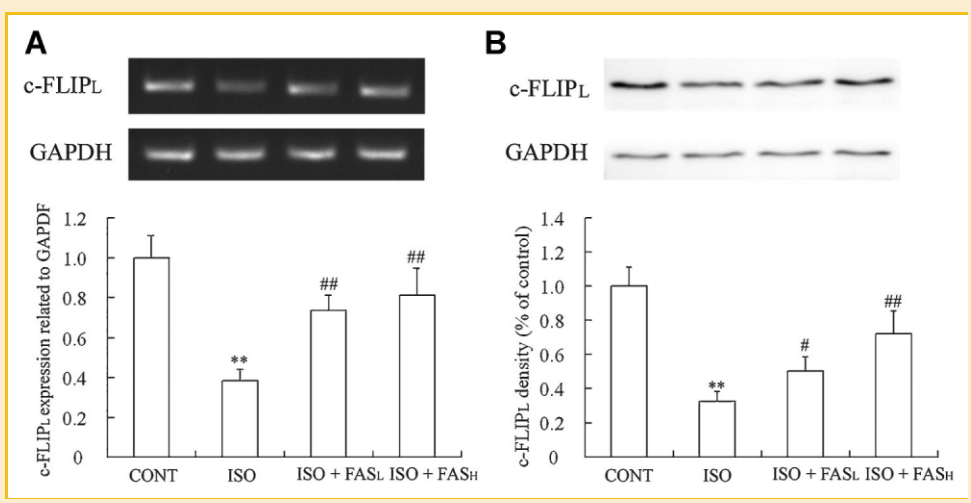

Fig. 7. Effects of fasudil on the c-$FLIP_L$ mRNA and protein expression in rat LV of the four indicated groups. A: Fasudil reduced ISO-induced c-$FLIP_L$ mRNA expression. After total RNA extraction, RT-PCR was performed as described in Materials and methods. Intensity of c-$FLIP_L$ was standardized to that of GAPDH. B: Fasudil reduced ISO-induced c-$FLIP_L$ protein expression as determined by immunoblotting. Intensity of c-$FLIP_L$ was standardized to that of GAPDH. Data are shown as the mean ± S.E.M. $^{**}P < 0.01$, vs. CONT group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. ISO group, n = 6.

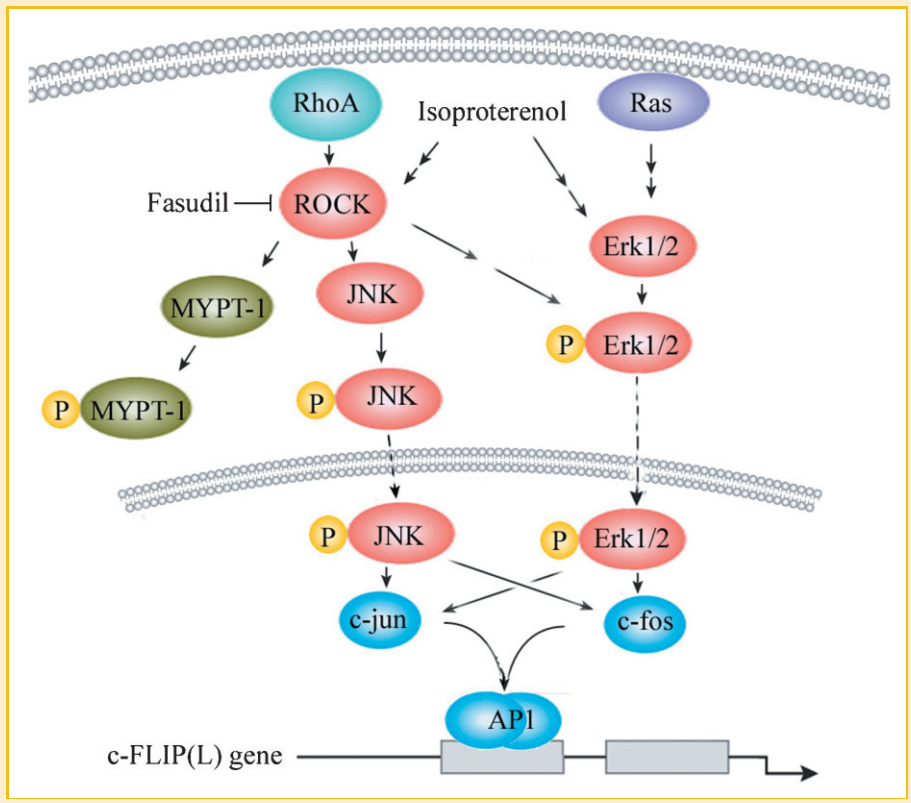

Fig. 8. Proposed signaling pathways involved in the protective effect of fasudil on ISO induced heart failure. RhoA/ROCK is activated by ISO and its activation is essential for heart failure induced by ISO. Fasudil is capable of suppressing ISO induced heart failure, which is associated with inhibition of JNK activation, ERK translocation to the nucleus, subsequent AP-1 (c-fos and c-jun) expression and up-regulation of c-FLIP$_L$ expression.

1997; Ventura et al., 2003]. It is well known that c-fos dimerizes with c-jun to form the AP-1 complex, a transcription factor which induces target gene expression by binding the AP-1 consensus site(s) in the promoter region. AP-1 is ubiquitously expressed in the brain and has been shown to be associated with a variety of physiological and pathophysiological states [Herdegen and Waetzig, 2001; Rylski and Kaczmarek, 2004], similar to its effect in the heart. Since AP-1 plays a pivotal role in the ERK/JNK signaling pathways [Fukushima et al., 2005] and overexpressions of c-jun and c-fos are common features of rodent hypertrophy [Saadane et al., 1999], we examined the effect of fasudil on c-fos and c-jun mRNA expression on ISO treated cardiomyocytes in this study. Our data showed that fasudil evidently suppressed the ISO-induced JNK/ERK signaling pathways and expression of their down-stream effectors c-fos and c-jun mRNA, suggesting that the inhibitory effects of fasudil on JNK activation, ERK translocation to the nucleus and subsequent c-fos and c-jun expression contributed to the inhibition of rat heart failure.

Extensive research is ongoing to elucidate the role of c-FLIP$_L$ as an inhibitor of apoptosis [Chang et al., 2002; Micheau et al., 2002; Boatright et al., 2004]. Schmitz et al. found that c-FLIP-deficient mice are prone to heart failure [Schmitz et al., 2004] and that c-FLIP is highly expressed in their hearts. Gilot et al. testified that the phosphorylated form of ERK1/2 is responsible for c-FLIP$_L$ induction [Gilot et al., 2005]. AP-1 binds to the c-FLIP$_L$ promoter, represses its transcriptional activity and reduces c-FLIP$_L$ mRNA and protein levels [Li et al., 2007]. RhoA signaling also has been shown to regulate AP-1 transcriptional activation in some cell types [Bolaman et al., 2005]. Taking this into consideration with our findings, we propose that the increase in ROCK by ISO can cause the up-regulation of ERK/JNK activation, which in turn induces AP-1 activation. Increased AP-1 activation would cause a decrease in c-FLIP$_L$ promoter activity. Thus, c-FLIP$_L$ is possibly an integral part of the ERK/JNK signaling pathway which may be the indispensable link between RhoA/ROCK pathway and c-FLIP$_L$ transcription. Furthermore, the effects of fasudil further confirmed the existence of this pathway. The up-regulation of c-FLIP$_L$ expression can be fully prevented by ERK inhibitors such as UO126, indicating that the ERK1/2 pathway may effectively control c-FLIP$_L$ expression levels [Gilot et al., 2005]. The low expression of c-FLIP$_L$ induced by ISO may also be a reason for myocardial damage. Since c-FLIP$_L$ can resist death receptor–induced apoptosis, the increased c-FLIP$_L$ expression suggests that it is involved in the protective effect of fasudil against ISO-induced cardiomyopathy.

In summary, the present study demonstrated that RhoA/ROCK is activated by ISO and that its activation is essential for heart failure induced by ISO (Fig. 8). Furthermore, fasudil is capable of suppressing ISO-induced heart failure, which is associated with inhibition of JNK activation, ERK translocation to the nucleus, subsequent AP-1(c-fos and c-jun) expression and up-regulation of

c-FLIP$_L$ expression. These results will help provide further insight into the molecular mechanisms underlying the beneficial effects of fasudil on heart diseases.

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