The Role of Rho Kinase and Autophagy in Fasudil Against Myocardial Ischemia/Reperfusion Injury Postprint

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Abstract

Objective: To investigate the changes in autophagy during fasudil (Fasudil)-mediated myocardial protection through Rho-kinase inhibition, and to analyze its role and potential mechanisms. Methods: Isolated rat hearts were perfused using the Langendorff apparatus. The left anterior descending coronary artery was ligated for 30 min to simulate regional myocardial ischemia, and the ligature was released to restore perfusion for 120 min to establish a myocardial ischemia/reperfusion (I/R) model. The experiment was divided into three groups: I/R group, fasudil (Fasudil) group, and fasudil + autophagy inhibitor wortmannin (Wort) group. Left ventricular hemodynamic parameters were continuously recorded. Coronary effluent was collected at 5 and 10 min of reperfusion to measure lactate dehydrogenase (LDH) content. RT-PCR was used to detect changes in mRNA expression of autophagy-related genes Atg5, Beclin1 and apoptosis-related genes Bax, Bcl-2. Western blotting was used to detect changes in caspase-3 protein expression. Results: Compared with the I/R group, fasudil significantly improved left ventricular developed pressure, maximum rates of left ventricular pressure rise and decline, and left ventricular work, reduced LDH release in coronary effluent during reperfusion, increased mRNA expression of Atg5 and Beclin1, elevated the Bcl-2/Bax mRNA ratio, and decreased caspase-3 protein expression. The autophagy inhibitor wortmannin (Wort) attenuated the protective effects of fasudil, inhibited the recovery of ventricular hemodynamic parameters, increased LDH release, decreased mRNA expression of Atg5 and Beclin1, reduced the Bcl-2/Bax ratio, and increased caspase-3 protein expression. Conclusion: Rho-kinase inhibition-induced myocardial protection triggered autophagy, and autophagy played a cardioprotective role, which may be associated with reduced apoptosis.

Full Text

Preamble

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Role of Autophagy in Fasudil-Induced Rho Kinase Inhibition for Protection Against Myocardial Ischemia-Reperfusion Injury in Rats

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Abstract

Objective: To investigate the changes in autophagy during fasudil-mediated Rho kinase inhibition in ischemic myocardium and to analyze its role and potential mechanisms. **Methods:** Isolated rat hearts were perfused using a Langendorff apparatus. The left anterior descending coronary artery was ligated for 30 minutes to simulate regional myocardial ischemia, followed by release of the ligation and 120 minutes of reperfusion to establish a myocardial ischemia/reperfusion (I/R) model. Hearts were randomly divided into three groups: I/R group, fasudil group, and fasudil+autophagy inhibitor wortmannin (Wort) group. Left ventricular hemodynamic parameters were continuously recorded. Coronary effluent was collected at 5 and 10 minutes of reperfusion to measure lactate dehydrogenase (LDH) content. RT-PCR was used to detect mRNA expression of autophagy-related genes Atg5 and Beclin1 and apoptosis-related genes Bax and Bcl-2, while Western blotting was performed to assess caspase-3 expression changes. **Results:** Compared with the I/R group, fasudil significantly improved left ventricular developed pressure, maximal rise/fall rate of left ventricular pressure, and left ventricular work, while reducing LDH release during reperfusion. Fasudil also increased Atg5 and Beclin1 mRNA expression, elevated the Bcl-2/Bax mRNA ratio, and decreased caspase-3 protein expression. The autophagy inhibitor wortmannin attenuated these protective effects of fasudil, suppressed recovery of ventricular hemodynamic indices, increased LDH release, decreased Atg5 and Beclin1 mRNA expression, reduced the Bcl-2/Bax ratio, and increased caspase-3 protein expression. **Conclusion:** Rho kinase inhibition confers myocardial protection by inducing autophagy, which plays a cardioprotective role likely associated with reduced apoptosis.

Keywords: heart; ischemia/reperfusion injury; autophagy; fasudil; wortmannin

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Introduction

Cardiovascular disease poses a serious threat to human life and health. While reperfusion therapy has improved cardiac function and achieved good therapeutic efficacy, it has also introduced a new problem—ischemia/reperfusion (I/R) injury. Investigating the mechanisms underlying I/R injury is crucial for preventing and treating clinical myocardial damage. Both Rho kinase and autophagy are involved in myocardial I/R injury. Rho kinase, a serine-threonine protein kinase family, plays an important role in I/R injury, and inhibiting Rho kinase can protect against myocardial ischemia/reperfusion injury [1-2].

Autophagy is currently a hot topic in disease mechanism research. Initially considered a highly conserved normal physiological process for degrading cytoplasmic components within lysosomes during evolution, autophagy has been shown in recent years to be involved not only in maintaining physiological homeostasis but also associated with aging, neurodegenerative diseases, and cardiovascular diseases [3-4]. The role of autophagy in myocardial I/R injury remains controversial. On one hand, enhanced autophagy may lead to cell death and exacerbate myocardial I/R injury [5]; on the other hand, autophagy may play a beneficial role in I/R injury [6-7]. Therefore, although numerous studies have shown that autophagy participates in myocardial I/R injury, its precise role remains undetermined.

Some studies have indicated a connection between Rho kinase and autophagy [8], but how autophagy occurs and what role it plays in fasudil-mediated Rho kinase inhibition against myocardial I/R injury have not been reported. Clarifying the role and potential mechanisms of autophagy could provide new therapeutic avenues for reducing myocardial I/R injury. In this study, we established an isolated rat heart I/R injury model to examine changes in autophagy-related gene Atg5 and Beclin1 mRNA expression during Rho kinase inhibition, thereby determining whether autophagy is involved in the cardioprotective effects of Rho kinase inhibition. We further applied an autophagy inhibitor to investigate the role of autophagy in fasudil-mediated cardioprotection and examined changes in apoptosis-related factors to analyze the relationship between autophagy and apoptosis in myocardial protection.

Materials and Methods

1.1 Experimental Animals and Materials

Healthy male Sprague-Dawley (SD) rats (specific pathogen-free grade, weighing 220-250 g, n=24) were provided by the Experimental Animal Center of Bengbu Medical College. Fasudil (Rho kinase inhibitor) was purchased from Tianjin Hongri Pharmaceutical Co., Ltd. The autophagy inhibitor wortmannin (Wort) was obtained from Sigma. Modified Krebs-Henseleit (K-H) solution contained: NaCl 118 mmol/L, KCl 4.7 mmol/L, KH PO 1.2 mmol/L, MgSO 1.2 mmol/L,

CaCl 2.5 mmol/L, NaHCO 25 mmol/L, glucose 11 mmol/L, pH 7.3-7.4. Trizol reagent for total RNA extraction was purchased from Invitrogen. Reverse transcription and PCR kits were from Fermentas. Primers were synthesized by Shanghai Sangon Biotech Engineering Services Co., Ltd.:

- Atg5 primers: forward 5' -AGT GGA GGC AAC AGA ACC-3' , reverse 5' -GAC ACG AAC TGG CAC ATT-3' , expected product length 205 bp
- Beclin1 primers: forward 5'-GAG CCA TTT ATT GAA ACT CGC CA-3' , reverse 5' -CCT CCC CGA TCA GAG TGA A-3' , expected product length 106 bp
- Bax primers: forward 5'-GGA TCG AGC AGA GAG GAT GG-3', reverse 5' -TGG TGA GTG AGG CAG TGA GG-3' , expected product length 464 bp
- Bcl-2 primers: forward 5' -CTG GTG GAC AAC ATC GCT CTG-3' , reverse 5' -GGT CTG CTG ACC TCA CTT GTG-3' , expected product length 228 bp
- -actin (internal control) primers: forward 5' -CTG TAT GCC TCT GGT CGT AC-3', reverse 5'-TGA TGT CAC GCA CGA TTT CC-3', expected product length 214 bp

Primary antibodies including mouse anti-rat -actin and rabbit anti-rat caspase-3 were from Cell Signaling Technology. HRP-labeled secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were from Affinity.

1.2 Isolated Rat Heart Ischemia/Reperfusion Model and Experimental Groups

Rats were anesthetized with 4% chloral hydrate, decapitated, and their hearts were rapidly excised and arrested in ice-cold water. Hearts were then perfused retrogradely through the aortic trunk using a Langendorff apparatus. K-H solution was perfused at constant pressure (76 mmHg), saturated with 95% O + 5% CO , and maintained at 37°C. The left atrium was incised and a latex balloon was inserted into the left ventricle. Intraventricular pressure was transmitted through a Teflon tube to a pressure transducer, and data were recorded using a Medlab biological signal acquisition and processing system.

After a 25-minute stabilization period, experimental interventions were performed. Hearts were randomly divided into three groups (n=8 each):

1. **Ischemia/Reperfusion (I/R) group:** The left anterior descending coronary artery was ligated for 30 minutes to induce regional myocardial ischemia, followed by 120 minutes of reperfusion after releasing the ligature.
2. **Fasudil group:** Same as I/R group, but treated with 30 mol/L fasudil from 10 minutes before ischemia through the first 10 minutes of reperfusion (total 50 minutes).
3. **Fasudil+Wort group:** Same as I/R group, but treated with both 30 mol/L fasudil and 100 nmol/L wortmannin during the same period (total

50 minutes).

1.3 Left Ventricular Function Indices

The latex balloon was filled with water to maintain left ventricular end-diastolic pressure (LVEDP) at 4-10 mmHg. The Medlab system recorded left ventricular developed pressure (LVDP), LVEDP, and maximal rise/fall rate of left ventricular pressure (±dp/dt). Left ventricular work was calculated as rate-pressure product (RPP), where RPP = LVDP × HR [9].

1.4 Measurement of Lactate Dehydrogenase (LDH) in Coronary Effluent

Coronary effluent was collected at 5 and 10 minutes of reperfusion. LDH content was measured by spectrophotometry and expressed in U/L.

1.5 RT-PCR Detection of Atg5, Beclin1, Bax, and Bcl-2 mRNA

Total RNA was extracted from left ventricular tissue using Trizol reagent. Three microliters of total RNA were used as template for cDNA synthesis with a reverse transcription kit. PCR amplification was performed using 1.5 L cDNA as template. Amplification conditions: initial denaturation at 95°C for 3 minutes, followed by cycles of (1) 95°C for 30 seconds, (2) annealing for 30 seconds at 64.5°C for Bax, 58°C for -actin, 56°C for Atg5, or 35 seconds at the appropriate temperature for Bcl-2, and (3) extension at 72°C for 30 seconds. Thirty cycles were performed with a final extension for 10 minutes. Four microliters of PCR products were subjected to agarose gel electrophoresis and visualized with ethidium bromide. Gel images were captured using a GIS gel imaging system, and band net optical density was analyzed semi-quantitatively using image analysis software. mRNA relative expression was expressed as the ratio of target gene to -actin net optical density (Atg5/ -actin, Beclin1/ -actin, Bcl-2/ -actin, Bax/ -actin), and the Bcl-2/Bax ratio was calculated.

1.6 Western Blotting Detection of Caspase-3 Expression

Reperfused myocardial tissue was collected and protein was extracted using a kit. Protein concentration was determined by BCA assay. Proteins were separated on 6-15% SDS-polyacrylamide gels and transferred to PVDF membranes at 200 mA. Membranes were blocked with 5% skim milk for 2 hours at room temperature, then incubated overnight at 4°C with primary antibodies (1:1000). After washing, membranes were incubated with HRP-labeled secondary antibodies for 2 hours at room temperature. Following washing, bands were visualized using a chemiluminescence kit on a CE Image Quant LAS 4000 mini imager and analyzed with Image J software.

Statistical Analysis

Data are expressed as mean ± standard deviation. Inter-group comparisons were performed using one-way ANOVA, with pairwise comparisons conducted using q-test. $P < 0.05$ was considered statistically significant.

Results

2.1 Changes in Left Ventricular Function

In the I/R group, LVDP decreased while LVEDP increased during ischemia and reperfusion, with ±dp/dt and RPP also decreasing. Compared with the I/R group, the fasudil group showed recovery of LVDP and ±dp/dt , reduced LVEDP, and increased RPP. In contrast, the autophagy inhibitor wortmannin attenuated these effects, decreasing LVDP, ±dp/dt , and RPP while markedly elevating LVEDP during reperfusion, indicating that autophagy inhibition weakened fasudil' s protective effects .

2.2 LDH Content in Coronary Effluent

Compared with the I/R group, fasudil significantly reduced LDH release from rat hearts at different time points during reperfusion. Co-treatment with wortmannin increased LDH release compared with the fasudil alone group [Figure 1: see original paper].

2.3 Expression of Atg5, Beclin1, Bcl-2, and Bax mRNA in Myocardial Tissue

The I/R group showed expression of autophagy-related genes Atg5 and Beclin1 and apoptosis-related factors Bcl-2 and Bax mRNA, indicating that both autophagy and apoptosis participate in myocardial I/R injury. Compared with the I/R group, the fasudil group exhibited increased Atg5 and Beclin1 mRNA expression, elevated Bcl-2 expression, and a higher Bcl-2/Bax ratio. In the fasudil+wortmannin group, Atg5 and Beclin1 mRNA expression and the Bcl-2/Bax ratio were decreased compared with the fasudil group , [Figure 2: see original paper], [Figure 3: see original paper].

2.4 Caspase-3 Protein Expression in Myocardial Tissue

Fasudil decreased caspase-3 protein expression compared with the I/R group. Wortmannin co-treatment increased caspase-3 protein expression compared with fasudil alone [Figure 4: see original paper], .

Discussion

Reperfusion therapy for cardiovascular disease introduces the challenge of ischemia/reperfusion (I/R) injury. While calcium overload, oxidative stress, and neutrophil infiltration are established mechanisms, the pathogenesis requires further elucidation. Rho kinase plays an important role in I/R injury, and inhibiting Rho kinase can protect the heart by activating the PI3K/Akt signaling pathway [10-11]. Our previous studies demonstrated that fasudil protects against myocardial I/R injury by inhibiting the Rho kinase signaling pathway, possibly through activating ALDH2 and suppressing apoptosis [12]. These findings suggest Rho kinase may participate in myocardial I/R injury through multiple mechanisms, but whether autophagy is involved and what role it plays remain unexplored.

In this study, we used the Langendorff apparatus to establish a myocardial I/R injury model and examined hemodynamic changes at different time points during ischemia and reperfusion. LVDP and +dp/dt reflect myocardial contractile function, while LVEDP and -dp/dt reflect diastolic function [13]. Our results showed that compared with the I/R group, fasudil treatment increased LVDP, ±dp/dt , and RPP while suppressing LVEDP elevation during reperfusion, indicating that fasudil-mediated Rho kinase inhibition improves both systolic and diastolic function after reperfusion.

Lactate dehydrogenase is a glycolytic enzyme whose early elevation in serum is a clinical marker for acute myocardial infarction. During I/R injury, increased cell membrane permeability leads to elevated LDH release, making coronary effluent LDH content an indicator of cellular damage [14]. We observed that fasudil reduced LDH release during reperfusion, suggesting that Rho kinase inhibition alleviates I/R injury.

Cell death occurs through necrosis, apoptosis, and autophagy, with both apoptosis and autophagy participating in I/R injury [15]. The cardioprotective effects of Rho kinase inhibition are associated with reduced apoptosis [1], but whether autophagy is also involved remains unclear. We therefore examined expression of autophagy-related genes Atg5 and Beclin1. Beclin1, the mammalian homolog of yeast autophagy gene Apg6/Vps30, is an important positive regulator of autophagy and serves as a key quantitative marker [16]. Atg5 is another crucial autophagy regulatory gene; the Atg5-Atg12 complex localizes to autophagosome membranes, promoting membrane expansion and autophagosome formation [17-18]. Our results showed increased Atg5 and Beclin1 mRNA expression in the fasudil group, suggesting that autophagy is induced during Rho kinase inhibition-mediated cardioprotection. Furthermore, application of the autophagy inhibitor wortmannin attenuated fasudil' s protective effects while suppressing autophagy, indicating that fasudil may exert cardioprotection by inducing autophagy.

Both apoptosis and autophagy participate in I/R injury, and crosstalk exists between these processes. Studies in HL-1 cardiomyocytes showed that downregulating autophagy-related genes Beclin1 or Atg5 increased I/R-induced apoptosis,

while Beclin1 overexpression enhanced autophagy and reduced apoptosis [19]. Hepatic ischemic preconditioning upregulates heme oxygenase-1, a key factor in autophagy and apoptosis, and induces autophagy via p38-MAPK signaling to engulf damaged mitochondria, thereby inhibiting apoptosis and protecting hepatocytes from I/R injury [20]. To investigate the relationship between autophagy and apoptosis following Rho kinase inhibition, we examined caspase-3 protein expression and Bcl-2/Bax mRNA levels. Caspase-3, an effector caspase downstream in the apoptosis cascade, is a critical executioner and convergence point for various apoptotic signals. Bax promotes apoptosis by binding to mitochondrial permeability transition pore proteins and releasing cytochrome c, whereas Bcl-2 inhibits Bax translocation to mitochondrial membranes, thereby suppressing apoptosis [21-23]. Our results showed that fasudil induced autophagy while decreasing caspase-3 expression and increasing the Bcl-2/Bax ratio, indicating reduced apoptosis. Conversely, wortmannin co-treatment suppressed autophagy while increasing caspase-3 expression and decreasing the Bcl-2/Bax ratio, suggesting enhanced apoptosis. These findings indicate that autophagy induction following Rho kinase inhibition may protect cardiomyocytes by reducing apoptosis.

In conclusion, in the isolated rat heart I/R injury model, fasudil-mediated Rho kinase inhibition induces autophagy, which contributes to cardioprotection by reducing apoptosis. However, the specific pathways through which Rho kinase inhibition induces autophagy and the detailed mechanisms of autophagy-apoptosis crosstalk require further investigation and represent the focus of our future work.

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Note: Figure translations are in progress. See original paper for figures.