Hirudin Protects Ang II-Induced Myocardial Fibroblasts Fibrosis by Inhibiting the Extracellular Signal-Regulated Kinase1/2 (ERK1/2) Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Myocardial fibrosis is closely related to all types of cardiovascular diseases. Hirudin is widely used in the prevention and treatment of cardiovascular diseases and cancers. In this study, we examined the potential role(s) and mechanism of hirudin in angiotensin II (Ang II)-induced myocardial fibrosis.

Material/Methods: The viability of myocardial fibroblasts, and reactive oxygen species (ROS) rates were measured respectively using cell counting kit-8 (CCK-8) and flow cytometry. Malondialdehyde (MDA) content, the activities of lactate dehydrogenase (LDH), and superoxide dismutase (SOD) were detected by the respective kits. The mRNA and protein levels of fibrosis-related factors were separately assessed by qRT-PCR and western blot.

Results: Our data revealed that hirudin suppressed the viability of myocardial fibroblasts, and that it relieved the proliferation induced by Ang II in a dose-dependent manner. We also found that hirudin reduced ROS production, LDH activity, and MDA content; however, it enhanced SOD activity. Moreover, while hirudin significantly downregulated the levels of matrix metalloproteinase-2 (MMP-2), MMP-9, fibronectin (FN), transforming growth factor beta 1 (TGF-β1), collagen-I (COL-I), and COL-III, it upregulated the expression level of tissue inhibitor of metalloproteinases-2 (TIMP-2). Furthermore, phosphorylated extracellular signal-regulated kinase1/2 (p-ERK1/2) was decreased by hirudin, compared to the Ang-II group.

Conclusions: Hirudin depressed Ang II-induced myocardial fibroblasts via inhibiting oxidative stress, regulating fibrosis-related factors, and repressing the ERK1/2 pathway.

MeSH Keywords: Angiotensin II • Endomyocardial Fibrosis • Hirudins • MAP Kinase Signaling System • Oxidative Stress

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Background

The incidence and mortality of cardiovascular diseases have been increasing in recent years. Cardiovascular diseases are characterized by high incidence, poor prognosis, and high mortality, which could seriously threaten the quality of life of patients [1,2]. At present, among cardiovascular diseases, heart failure has become the main cause of death. Heart failure is the end stage of the development of various cardiovascular diseases [3]. Ventricular remodeling is now considered an important pathological process of heart failure, and interstitial fibrosis plays an important role in ventricular remodeling [4]. Myocardial fibrosis is not only closely associated with all types of cardiovascular diseases such as dilated cardiomyopathy, hypertrophic cardiomyopathy, diabetic cardiomyopathy, and hypertensive heart disease [5], but myocardial fibrosis can lead to serious complications, for example, sudden cardiac death, heart failure, and arrhythmia [6–8]. Therefore, the prevention and reversal of myocardial fibrosis is of critical importance to the clinical treatment of cardiovascular disease.

Hirudin was first extracted from hirudo (a genus of leeches) in 1904, and it was the first anticoagulant used in humans [9]. Since then, the study of hirudin has been the focus of clinic studies [10–12]. Hirudin is composed of 65 amino acid residues, and its molecular weight is 7 kD. Its structure has 3 disulfide bonds at the N-terminus, which enables the N-terminal peptide chain to form a dense core cyclic peptide structure. The C terminal is rich in acidic amino acid residues. A special sequence located in the middle of peptide chain consists of Pro-1ys-Pro that is not degraded by the general protease [12–14]. Hirudin has the features of anticoagulant and anti-cancer function due to its structural features, which enable Hirudin to be widely used in the prevention and treatment of cardiovascular and cerebrovascular diseases and cancers [10,11,15]. Nevertheless, to the best of our knowledge, there is no relevant articles explaining the relation between hirudin and myocardial fibrosis.

For decades, mitogen-activated protein kinases (MAPK) signal transduction pathway has attracted enormous attention from researchers [16,17]. Extracellular signal-regulated kinase1/2 (ERK1/2) signal transduction pathway is the main pathway of the MAPK system, which is a cascade reaction of receptor tyrosine kinase and cytoplasmic protein kinase activated by a small GTP binding protein junction [18,19]. This pathway can transduce extracellular stimulation signals into the nucleus and cause biological responses, such as cell proliferation, differentiation, oxidative stress, and apoptosis [18,20]. The ERK1/2 pathway has been found in the process of cardiovascular disease and in relation to the occurrence and development of tumors [21–23].

In this study, we explored the potential effect of hirudin on myocardial fibroblast fibrosis induced by angiotensin II (Ang II). We further analyzed whether the potential mechanism regulated the ERK1/2 pathway or not.

Material and Methods

Cell culture

Murine myocardial fibroblasts were purchased from Jining Shiye Biological Technology Co., Ltd. (Shanghai, China). Cells were inoculated in complete Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Shanghai, China) that contained 10% fetal bovine serum (FBS; Haling, Shanghai, China) and 1% penicillin-streptomycin (Leagene, Beijing, China) in a humidified incubator with 5% CO_2 (HZ-80B-II; LNB Instrument, Shanghai, China) at 37°C.

Cell counting kit-8 (CCK-8) assay

Cell counting kit-8 (CCK-8, MSK, Wuhan, China) assays were performed to analyze cell viability following the manufacturer instructions. To explain further, cells were inoculated in 96-well plates (3×10^3 cell/well) for 24 hours. After being cultured, cells were treated respectively with phosphate buffered saline (PBS) (control) and hirudin (10, 20, 40, 80, 160, and 320 µg/mL) for 12, 24, and 48 hours. Other cells were treated independently with PBS (control), Ang II 10^{-6} mol/L, hirudin 20 µg/mL+Ang II 10^{-6} mol/L (Hirudin1+Ang II); Ang II 10^{-6} mol/L, hirudin 40 µg/mL+Ang II 10^{-6} mol/L (Hirudin2+Ang II); and hirudin 80 µg/mL+Ang II 10^{-6} mol/L (Hirudin3+Ang II) for 12, 24, and 48 hours. Next, CCK-8 reagent was added to the cells, which were then cultured in an incubator for 4 hours. Absorbance was analyzed at 450 nm using a light absorption microplate reader (Multistan FC; Thermo Fisher, USA).

Reactive oxygen species (ROS) assay

Reactive oxygen species (ROS) assay kit (Yeasen, Shanghai, China) was used to examine the rate of ROS according to instructions of the manufacturer. After administration to cells, cells were incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 25 min. Then cells were washed with PBS 3 to 4 times. ROS was investigated by flow cytometer (TMS-2050; Taomsun, Suzhou, Jiangsu, China).

Lactate dehydrogenase (LDH) assay

The LDH assay kit was used to detect LDH (Beyotime, Shanghai, China) following manufacturer's instructions. To be more specific, after administration to cells, cells were digested with trypsin and then centrifuged at 800 g for 3 min. Next, the supernatant was added to 96-well plates. Then 60 µL LDH working fluid was added into each well prior to cells incubation at

room temperature in the dark for 25 min. Absorbance at 490 nm was determined by a light absorption microplate reader.

Malondialdehyde (MDA) assay

Lipid peroxidation malondialdehyde (MDA) assay kit (Beyotime, Shanghai, China) was used to evaluate MDA activity under the guidance of the manufacturer. After cell treatment, the cells were crushed on ice by an ultrasonic cell breaker (Sonifier® SFX150; HQI, Shanghai, China). Then the cell liquid was mixed and heated in a 100°C boiling water bath for 15 min. The mixture was cooled to room temperature, and then was centrifuged at 800 g for 5 min. Absorbance at 532 nm was determined by a light absorption microplate reader.

Superoxide dismutase (SOD) assay

The superoxide dismutase (SOD) assay kit with WST-8 was performed to identify the activity of SOD (Beyotime, Shanghai, China) according to the manufacturer's instructions. After cell treatment, the cells were crushed on ice by an ultrasonic cell breaker. Then WST-8 reagent was added to cell suspension. After the reaction started working, fluid was added to the suspension. Subsequently, the suspension was incubated at 37°C for 25 min. The OD value at 450 nm was measured using a light absorption microplate reader.

Quantitative real-time PCR (qRT-PCR)

The samples were harvested, and then total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific, Shanghai, China). RNA was used to synthesize cDNA by applying cDNA synthesis kit (Promega, Beijing, China). RNA 1 μg, primer Olidod T 1 μL, M-MLV reverse transcriptase (Promega, USA), and nuclease-free water were mixed to 5 μL. The reaction conditions were as follows: 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. Then cDNA was amplified by SYBR Premix Taq™ II kit (Takara, Beijing, China). The reaction system (50 uL) contained the following elements: 25 μL SYBR Green Mix, 19 μL distilled water, 4 μL cDNA, and 1 μL forward/reverse primer. The amplification conditions were as follows: 95°C for 5 min; 35 cycles at 95°C for 15 sec; 62°C for 35 sec; 75°C for 30 sec. β-actin was considered as internal control. The $2^{-\Delta\Delta CT}$ method was used to quantify the expression level of target genes. The primers used were listed in Table 1.

Western blot

Total proteins were harvested and lysed with high RIPA lysis buffer (Leagene, Beijing, China). Proteins were separated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was bound to the PVDF membrane (Hongda; Zhuzhou, Guangdong China). Subsequently,

Table 1. Sequences of the primers.

Primer name	Sequence (5'-3')	Product size (bp)
MMP-2-forward	CCTTCACTTTCCTGGGCAAC	230
MMP-2-reverse	GGTGTAGGTGTAGATCGGGG	
MMP-9-forward	CCTGTGTGTTCCCGTTCATC	203
MMP-9-reverse	CAGGAAGACGAAGGGGAAGA	
TIMP-2-forward	GGAAATGCCGCAGATATCCG	239
TIMP-2-reverse	CAGGGAAACACTGTGCACAC	
FN-forward	TTGTTCGGTGGAGTAGACCC	220
FN-reverse	GTGCCAGTGGTCTCTTGTTG	
TGF-β1-forward	TCAGACATTCGGGAAGCAGT	243
TGF-β1-reverse	TCGAAAGCCCTGTATTCCGT	
COL-I-forward	GGCAAAGATGGAGAAGCTGG	214
COL-I-reverse	GGAAACCTCTCTCGCCTCTT	
COL-III-forward	CTTTGTGCAAAAGGGGAGCT	223
COL-III-reverse	TGGGTTGGGGCAGTCTAATT	
β-actin-forward	GTGGGAATGGGTCAGAAGGA	226
β-actin-reverse	TCATCTTTTCACGGTTGGCC	

5% skimmed milk was used to seal membranes for 1.5 hours. The membranes were incubated with anti-matrix metalloproteinase-2 (MMP-2) (AmyJet Scientific, 12015-01, 1: 2000), anti-MMP-9 (R&D, AF909-SP, 1: 1000), anti-tissue inhibitor of metalloproteinases-2 (TIMP-2) (R&D, AF971, 1: 1200), anti-fibronectin (FN) (Abcam, ab2413, 1: 800), anti-transforming growth factor beta 1 (TGF-β1) (R&D, FAB766G, 1: 700), anti-collagen-I (COL-I) (Abcam, ab90395, 1: 1000), anti-collagen III (COL-III) (Abcam, ab7778, 1: 800), anti-p-ERK1/2 (CST, 8544, 1: 1000), anti-ERK1/2 (CST, 9102, 1: 700), and anti-β-actin (Abcam, ab8226, 1: 2000) at 4°C for 24 hours. The next day, membranes were incubated with the secondary antibodies (HRP mouse anti-rabbit, Invitrogen, BA1034, 1: 7000; HRP goat anti-mouse IgG, Abcam, ab7064, 1: 8000; HRP mouse ant-goat IgG, Invitrogen, BA1074, 1: 7000) at 37°C for 60 min. The protein was exposed via the ECL chromogenic solution (Biodragon, Beijing, China).

Statistical analysis

Prism GraphPad 6.0 was adopted for statistical analysis. All experimental data were presented as mean ±SD. One-way ANOVA following Turkey's test was performed to analyze the differences among groups. The experiment was carried out for at least 3 times. $P < 0.05$ was regarded as statistically significant.
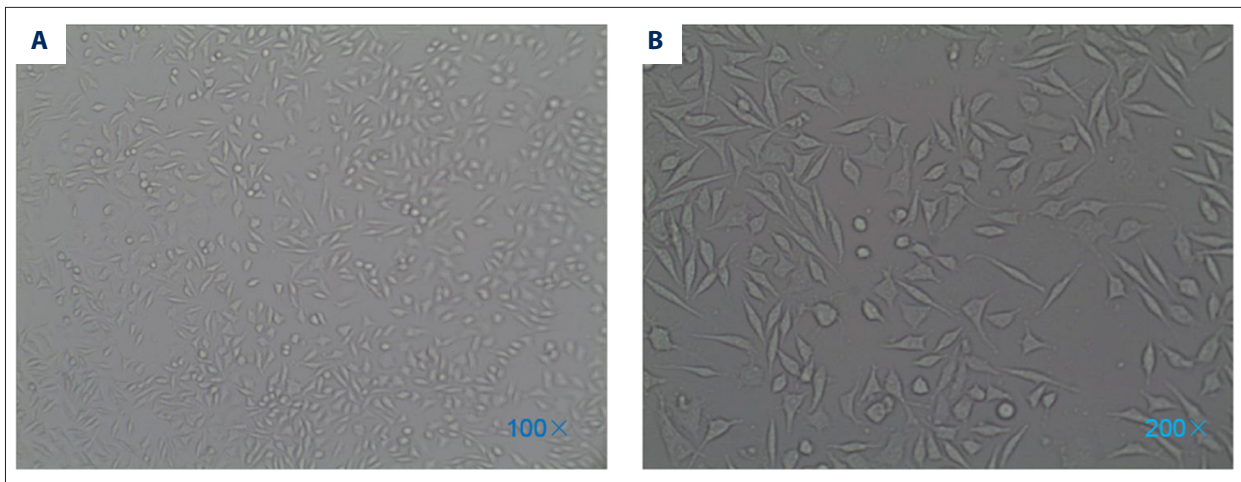
Figure 1. Morphological characteristic of myocardial fibroblasts. (**A, B**) Cells were cultured in DMED containing 10% FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37°C for 24 hours. Next day, the cells were observed and photographed under a microscope (100× and 200×).
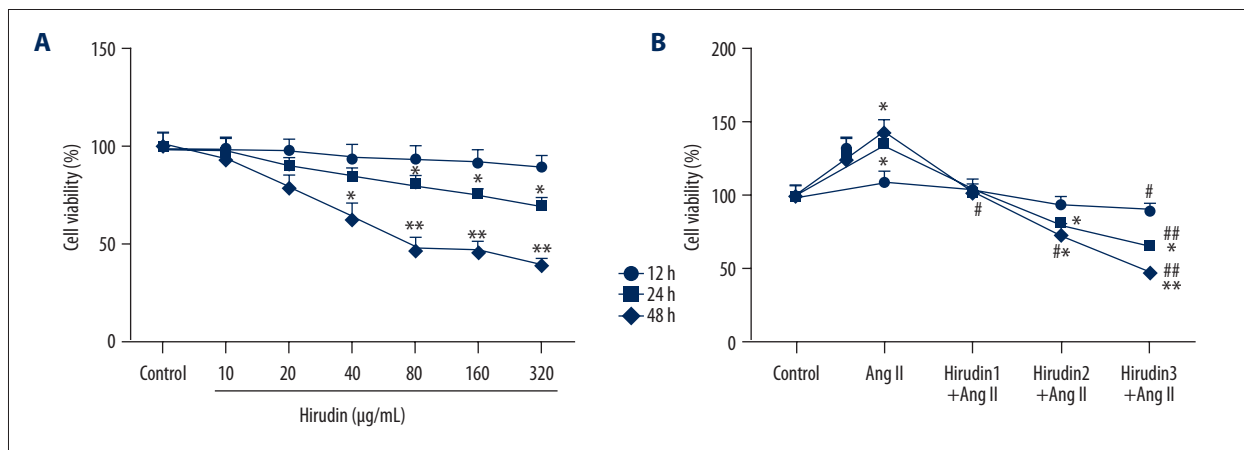

Figure 2. Hirudin repressed the viability of Ang-II induced myocardial fibroblasts. (**A**) Cells were treated respectively with PBS (control), hirudin (10, 20 40, 80, 160, and 320 µg/mL) for 12, 24, and 48 hours. CCK-8 was used to detect cell viability. * $P<0.05$, ** $P<0.01$ *vs.* control. (**B**) Cells were treated respectively with PBS (control), Ang II 10^{-6} mol/L, hirudin 20 µg/mL+Ang II 10^{-6} mol/L (Hirudin1+Ang II), hirudin 40 µg/mL+Ang II 10^{-6} mol/L (Hirudin2+Ang II) and Hirudin 80 µg/mL+Ang II 10^{-6} mol/L (Hirudin3+Ang II) for 12, 24 and 48 hours. The viability of cells was measured by CCK-8. * $P<0.05$, ** $P<0.01$ *vs.* control. # $P<0.05$, ## $P<0.01$ *vs.* Ang-II. Ang II – angiotensin II; PBS – phosphate buffered saline; CCK-8 – cell counting kit-8.

Results

Morphological characteristic of myocardial fibroblasts

We placed the cells under a light microscope to observe the morphology of the cells and took pictures after the cells were cultured for 24 hours. All cells were observed to adhere to the wall. Under the microscope, most of the myocardial fibroblasts were shown as spindle-shaped with a transparent cytoplasm (Figure 1).

Hirudin repressed the viability of Ang II-induced myocardial fibroblasts

CCK-8 was used to determine cell viability. The data showed that hirudin markedly inhibited cell viability at 24 hours and 48 hours in a dose-dependent manner. The cell viability was lower than 50% when cells were treated with 160 µg/mL and 320 µg/mL of hirudin for 48 hours. The concentrations of 20 µg/mL, 40 µg/mL, and 80 µg/mL hirudin were selected for later study as the IC50 of hirudin was 80 µg/mL at 48 hours. Additionally, we observed that Ang II significantly promoted the proliferation of cells at 24 hours and 48 hours. However, hirudin obviously reversed the role of Ang II in inducing cell
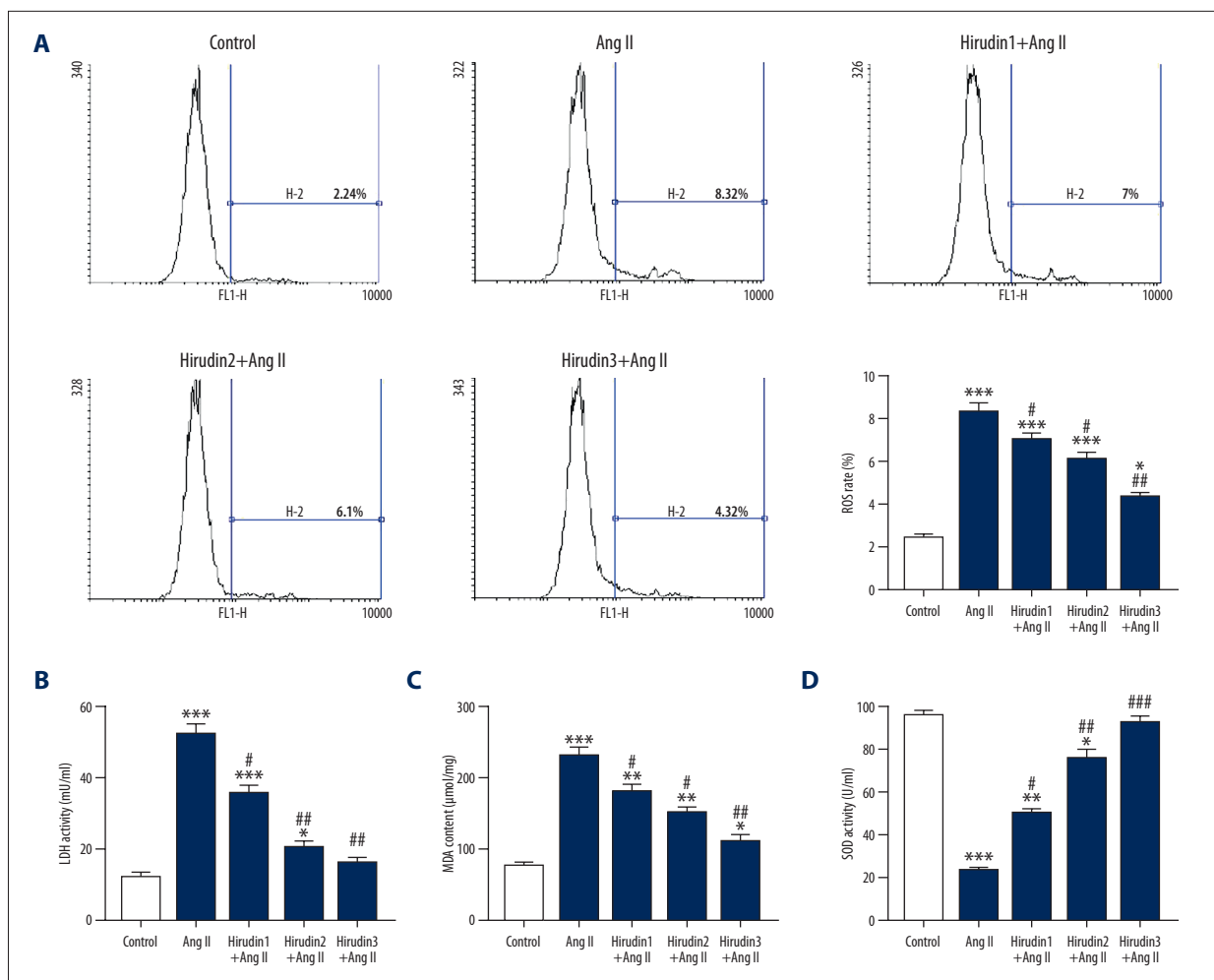
Figure 3. Hirudin decreased the oxidative stress of Ang II-induced myocardial fibroblasts. (**A**) The rate of ROS was analyzed using ROS detection kit. (**B**) The activity of LDH was measured by LDH assay kit. (**C**) The content of MDA was assessed by lipid peroxidation MDA assay kit. (**D**) The activity of SOD was detected by SOD assay kit. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* control. # $P<0.05$, ## $P<0.01$, ### $P<0.001$ *vs.* Ang II. ROS – reactive oxygen species; LDH – lactate dehydrogenase; MDA – malondialdehyde; SOD – superoxide dismutase, Ang II – angiotensin II.

proliferation at 24 hours and 48 hours. Meanwhile, the effect of hirudin at 48 hours was more obvious than that at 24 hours. Thus, the cells were treated with hirudin for 48 hours in subsequent experiments (Figure 2, $P<0.05$).

Hirudin decreased the oxidative stress of Ang II-induced myocardial fibroblasts

We studied the level of ROS, the activities of LDH and SOD, and MDA content for the purpose of exploring the effect of hirudin on the oxidative stress of Ang II-induced myocardial fibroblasts. Study results revealed that first, Ang II significantly increased the rate of ROS, the activity of LDH, and the MDA content. Second, in comparison with the Ang II group, the production of ROS in the Hirudin1+Ang II group, the Hirudin2+Ang II group and the Hirudin3+Ang II group were reduced respectively

by 1.32%, 2.22%, and 4%. Finally, we found that hirudin markedly attenuated LDH activity and MDA content and enhanced the activity of SOD in a concentration-dependent manner (Figure 3, $P<0.05$).

Hirudin regulated fibrosis-related factors in Ang II-induced myocardial fibroblasts

The effects of hirudin on fibrosis-related factors was studied by performing qRT-PCR and western blot on the evaluation of the relative mRNA and protein levels of MMP-2, MMP-9, TIMP-2, FN, TGF-β1, COL-I, and COL-III. Our qRT-PCR data showed that Ang II significantly upregulated levels of MMP-2, MMP-9, FN, TGF-β1, COL-I, and COL-III, while TIMP-2 level was observed to be downregulated by Ang II. We identified that hirudin considerably reduced the expression levels of MMP-2,
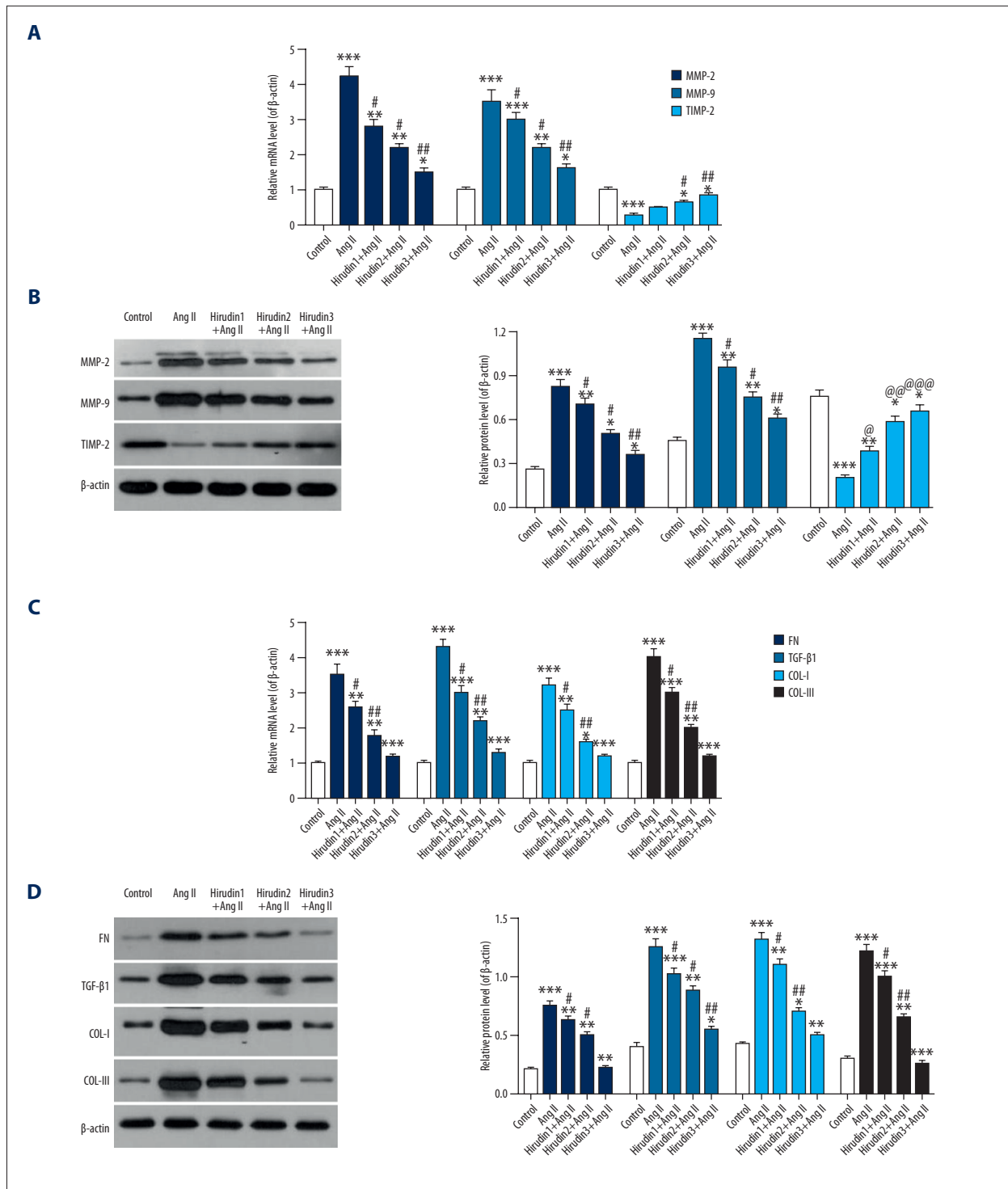
Figure 4. Hirudin regulated fibrosis-related factors in Ang II-induced myocardial fibroblasts. (**A**) The relative mRNA levels of MMP-2, MMP-9, TIMP-2 was evaluated by qRT-PCR. (**B**) The relative protein levels of MMP-2, MMP-9, TIMP-2 was examined by western blot. (**C**) qRT-PCR was performed to determine the mRNA levels of FN, TGF-β1, COL-I and COL-III. (**D**) Western blot was carried out to detect the protein levels of FN, TGF-β1, COL-I and COL-III, and normalized to β-actin expression. Gray value was detected and counted by quality one. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* control. # $P<0.05$, ## $P<0.01$, ### $P<0.001$, *vs.* Ang II.
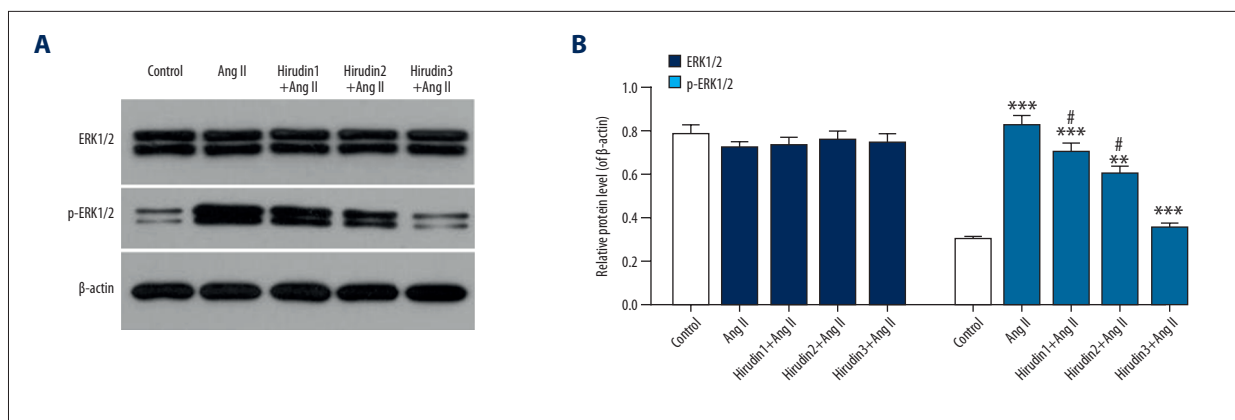
Figure 5. Hirudin downregulated ERK1/2 pathway in Ang II-induced myocardial fibroblasts. (**A**) The proteins expression levels of ERK1/2 and p-ERK1/2 were examined by western blot. (**B**) Quantification of protein expression was carried out with GraphPad prism 7.0. β-actin was used as internal control. The gray value was evaluated and calculated by quality one. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* control. # $P<0.05$, ## $P<0.01$, ### $P<0.001$, *vs.* Ang II.

MMP-9, FN, TGF-β1, COL-I, and COL-III, but it increased TIMP-2 expression in a dose-dependent manner, compared to Ang II group. Furthermore, western blot results observed that the expression trend of all proteins was consistent with mRNA (Figure 4, $P<0.05$).

Hirudin downregulated ERK1/2 pathway in Ang II-induced myocardial fibroblasts

To investigate the role of hirudin on the ERK1/2 pathway, western blot was carried out to assess the relative proteins of ERK1/2 and p-ERK1/2. Our results showed that in contrast to the control group, Ang II considerably improved the phosphorylation level of ERK1/2, and level of p-ERK1/2 was increased approximately 2.73 times. However, p-ERK1/2 levels were respectively declined about 15%, 27%, and 48% in the Hirudin1+Ang II group, the Hirudin2+Ang II group and the Hirudin3+Ang II group, compared to Ang II group (Figure 5, $P<0.05$).

Discussion

Ang II may lead to myocardial fibrosis by promoting fibroblast proliferation and increasing collagen synthesis and deposition. Thus, Ang II can indirectly promote myocardial fibrosis and accelerate myocardial remodeling [24]. Moreover, Ang II could increase the MAPK activity and DNA synthesis of cardiac fibroblast, therefore stimulating mRNA and protein expression of TGF-β1, laminin, and FN. Thus, Ang II would ultimately contribute to the development of cardiac interstitial fibrosis [25]. Applying Ang II to induce cardiac myocyte fibrosis has been diffusely used *in vitro* and *in vivo* experiments [26,27]. Therefore, Ang II was chosen to induce myocardial fibrosis in this study.

One of the pivotal causes of myocardial fibrosis is oxidative stress, which refers to the imbalance between oxidation and antioxidant activity in human body [28]. The over-accumulation of ROS has been suggested to trigger oxidative stress [29]. MDA is one of the most important products of membrane lipid peroxidation; LDH can accelerate oxidation, and SOD is an antioxidant enzyme that has an antioxidation effect [30,31]. High levels of ROS, LDH, and MDA but low SOD activity have been observed in myocardial fibrosis [32–34]. In our study, we found that Ang II accelerated oxidative stress of myocardial fibroblasts by increasing levels of ROS, LDH, and MDA, and by attenuating SOD activity.

Increasing evidence validates that patients with cardiovascular disease can be treated and controlled with hirudin [35–37]. Researchers has found that hirudin contributed to suppressing growth of fibroblasts [38], and to eliminating ROS accumulation [39]. Our results demonstrated that hirudin decreased viability of Ang II-induced myocardial fibroblasts, depressed ROS accumulation, LDH activity and MDA content, and enhanced SOD activity. Thus, we concluded that hirudin played an important role in relieving Ang II-induced oxidative stress.

Many studies have indicated that myocardial fibrosis was regulated by various factors, including MMPs, TIMPs, COL, FN, and TGF-β [40–43]. COL-I and COL-III are considered to be biomarkers of myocardial fibrosis, and increase significantly in myocardial fibrosis patients [42,43]. The dynamic balance between MMPs and TIMPs in the extracellular matrix are broken during myocardial fibrosis [44]. As the most important pro-fibrogenic growth factor, TGF-β1 has been found to induce the synthesis of extracellular matrix proteins, which synthesizes and secretes MMP2, ultimately mediating myocardial remodeling [41]. Thus, we explored the effect of hirudin on the expressions of these factors in Ang II-induced myocardial fibrosis. Our data

revealed that hirudin significantly inhibited the expression of MMP2, MMP9, FN, TGF-β1, COL-I, and COL-III, and elevated TIMP-2 expression. These results suggested that hirudin alleviated Ang II-induced fibrosis by regulating these factors.

ERK1/2 is phosphorylated with the progression of cardiomyocytes fibrosis. Activated ERK1/2 will then initiate the activation of downstream signaling [45,46]. In the present study, we supposed that hirudin might block Ang II-induced myocardial fibrosis via regulating the ERK1/2 pathway. We found that hirudin notably decreased phosphorylation level of ERK1/2 that was enhanced by Ang II. Therefore, we conclude that hirudin reversed Ang II-induced myocardial fibrosis via inhibiting the ERK1/2 pathway.

Conclusions

In summary, Ang II significantly stimulated myocardial fibroblasts fibrosis, it caused oxidative stress, and activated the ERK1/2 signal in cardiomyocytes. Hirudin clearly alleviated Ang II-induced fibrosis. Therefore, hirudin may be developed as a drug for the prevention and treatment of heart disease caused by myocardial fibrosis.

Conflict of interest

None.

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