

## Relaxin Inhibits Angiotensin II-Induced Cardiac Fibrosis by Activating NO/cGMP Signaling Pathway

### ABSTRACT

**Background:** Cardiac fibrosis, a key contributor to heart failure, is driven by the activation of cardiac fibroblasts (CFs), often induced by angiotensin II (Ang II). Relaxin, a peptide hormone, has been reported to counteract fibrotic processes. This study aims to investigate the anti-fibrotic effects of relaxin on Ang II-induced CF activation, with a focus on the involvement of the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling pathway.

**Methods:** Primary CFs were isolated and treated with Ang II to induce fibrotic activation. Relaxin was used to assess its antifibrotic effects. Inhibitors of the NO/cGMP pathway, NG-nitro-L-arginine methyl ester (L-NAME) (a nitric oxide synthase inhibitor) and 1H-(1,2,4)-Oxadiazolo-(4,3-a) quinoxalin-1-one (ODQ) (a guanylyl cyclase inhibitor), were co-administered to examine their effects on relaxin-mediated inhibition. Proliferation and migration were assessed using 5-Ethynyl-2'-deoxyuridine incorporation and Transwell assays. Western blot analysis was conducted to measure the expression of alpha-smooth muscle actin ( $\alpha$ -SMA), collagen I, and collagen III, key markers of fibroblast activation. Nitric oxide, cGMP, total nitric oxide synthase (TNOS), and inducible nitric oxide synthase (iNOS) levels were measured in the culture media.

**Results:** Ang II significantly increased CF proliferation, migration, and the expression of fibrosis markers  $\alpha$ -SMA, collagen I, and collagen III. Relaxin treatment markedly reduced these effects. Inhibition of the NO/cGMP pathway by L-NAME or ODQ partially reversed relaxin's suppressive effects on CF proliferation and migration. Relaxin restored Ang II-induced reductions in NO, cGMP, and TNOS levels, while iNOS levels remained largely unchanged, except for a reduction in the L-NAME group.

**Conclusion:** Relaxin attenuates Ang II-induced cardiac fibroblast activation and fibrosis primarily through the NO/cGMP signaling pathway.

**Keywords:** Relaxin, fibrosis, proliferation, migration, NO, cGMP

### INTRODUCTION

Cardiac fibrosis is a pathological condition characterized by the excessive accumulation of extracellular matrix (ECM) components, particularly collagen, within the myocardium.<sup>1</sup> This process results in the stiffening of cardiac tissue and impairment of heart function, which contributes to a range of cardiovascular conditions, including arrhythmias, cardiomyopathies, myocardial ischemia, and heart failure.<sup>2-4</sup> The underlying cause of cardiac fibrosis is often the activation of cardiac fibroblasts (CFs), which differentiate into myofibroblasts in response to stimuli such as inflammation, increased cardiac workload, and cardiomyocyte death.<sup>5</sup> Among these stimuli, angiotensin II (Ang II), a key effector of the renin-angiotensin-aldosterone system (RAAS), plays a central role by promoting CF proliferation, migration, and collagen production, particularly types I and III collagen.<sup>6</sup> This fibrotic remodeling further exacerbates the progression of heart failure and other cardiovascular diseases, making cardiac fibrosis a critical target for therapeutic intervention.

Despite the significant impact of cardiac fibrosis on heart function, current anti-fibrotic treatments remain limited and largely nonspecific. Progress in developing

### ORIGINAL INVESTIGATION

Jie Liu<sup>1</sup> 

Defeng Pan<sup>2</sup> 

Yuanyuan Luo<sup>2</sup> 

Wanling Wu<sup>2</sup> 

Tingbo Jiang<sup>1</sup> 

<sup>1</sup>Department of Cardiology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China

<sup>2</sup>Department of Cardiology, The Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, China

#### Corresponding author:

Tingbo Jiang  
✉ jtbsdfyy@163.com

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new antifibrotic therapies has been slow due to an incomplete understanding of the molecular mechanisms driving fibrosis.<sup>7-9</sup> As a result, elucidating these mechanisms and identifying targeted antifibrotic strategies are essential for improving the treatment and prevention of fibrotic heart diseases.

Relaxin, a hormone primarily known for its role in human reproduction, is also recognized for its therapeutic potential in treating cardiac fibrosis and myocardial remodeling.<sup>10,11</sup> Experimental studies have shown that relaxin is effective in reducing fibrosis by inhibiting the activation of CFs and the synthesis of collagen.<sup>12-14</sup> Furthermore, clinical studies have demonstrated that serelaxin, a recombinant form of relaxin, significantly reduces pulmonary congestion and myocardial damage, supporting its therapeutic potential in the treatment of heart disease.<sup>15-17</sup> The antifibrotic effects of relaxin are mediated, in part, through its interaction with the relaxin family peptide receptor 1 (RXFP1), which inhibits CF activation and collagen production.<sup>11,18,19</sup> Additionally, relaxin has been shown to modulate vasodilation, inflammation, and oxidative stress, all of which contribute to the fibrotic process.<sup>20</sup>

One of the primary mechanisms through which relaxin exerts its antifibrotic effects is by activating the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling pathway.<sup>21</sup> This pathway plays a critical role in regulating NO biosynthesis and NO synthase (NOS) expression, which are essential for modulating fibrosis in various tissues.<sup>22</sup> Studies have demonstrated that relaxin enhances NO production, upregulates NOS expression, and activates the NO/cGMP pathway, which contributes to its ability to reduce fibrosis in non-cardiac tissues.<sup>23</sup> Despite these findings, the precise mechanisms through which relaxin inhibits Ang II-induced fibrosis in CFs remain unclear.

In this study, we aim to investigate the effects of relaxin on Ang II-induced CF activation and to explore the involvement of the NO/cGMP signaling pathway in mediating these effects. By elucidating the molecular mechanisms underlying

relaxin's antifibrotic actions, we seek to provide insights into its potential therapeutic application in the treatment of cardiac fibrosis. Understanding how relaxin modulates the NO/cGMP pathway may offer new avenues for the development of targeted therapies to prevent or reverse the fibrotic processes that contribute to heart failure and other cardiovascular diseases.

## METHODS

### Animals and Reagents

Neonatal Sprague–Dawley (SD) rats were sourced from a certified animal center. A total of  $n=60$  rats were used in the study. The weights of the rats were standardized, with each rat weighing between 7 and 10 g at the time of surgery. Recombinant human relaxin-2 (relaxin) was acquired from a commercial supplier. Angiotensin II, methyl thiazolyl tetrazolium (MTT), NG-nitro-L-arginine methyl ester (L-NAME), 1H-(1,2,4)-Oxadiazolo-(4,3-a) quinoxalin-1-one (ODQ), and dimethylsulphoxide (DMSO) were sourced from Sigma-Aldrich. 5-Ethynyl-2'-deoxyuridine (EdU) was sourced from Ribobio Biotechnology. Type I and type III collagen and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibodies were sourced from Santa Cruz Biotechnology Inc. Vimentin, goat anti-rabbit IgG (H&L), Tetramethylrhodamine isothiocyanate (TRITC) antibody, and Bicinchoninic Acid (BCA) protein assay kit were purchased from Bioworld Inc. Enzyme-linked immunosorbent assay (ELISA) kits for cGMP were purchased from a Western Tang. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 0.25% trypsin were sourced from Gibco BRL. Nitric oxide and NOS reagent kits were obtained from Jiancheng Bioengineering Institute. Analytical grade reagents were used throughout the study.

### Isolation and Culture of Cardiac Fibroblasts

Neonatal rat CFs were prepared for culture using the following protocol. First, cardiac ventricles were isolated from 1- to 3-day-old SD rats. The ventricular tissue was minced into small pieces approximately 1 mm<sup>3</sup> in size in an ice-cold phosphate-buffered saline (PBS). The tissue samples were then digested with 0.08% trypsin and 0.08% collagenase I in PBS with gentle agitation for 5 minutes at 37°C. This digestion process was repeated 5-7 times until the tissues were fully digested. The cell suspensions were pooled and centrifuged at 1000 rpm for 5 minutes, then resuspended in DMEM containing 10% FBS. The cells were allowed to adhere to tissue culture plates in a 5% CO<sub>2</sub> incubator at 37°C. Weakly adherent or non-adherent cells were rinsed off and discarded after 1 hour. Fresh DMEM supplemented with 10% FBS was then added to the pre-plated CFs. When cells reached 80% confluency, they were digested with trypsin and replated.<sup>24</sup> Cells from the second and third passages were used for all experiments. Cardiac fibroblasts were identified by positive staining for the fibroblast marker vimentin and negative staining for  $\alpha$ -SMA, as verified by immunofluorescence. The subsequent experiments were assigned into 8 groups: control (Con), Ang II, relaxin, Ang II+relaxin, Ang II+L-NAME, Ang II+L-NAME+relaxin, Ang II+ODQ, and Ang II+ODQ+relaxin. NG-nitro-L-arginine methyl ester was used as a NOS inhibitor, while ODQ was employed as a

## HIGHLIGHTS

- Relaxin inhibits cardiac fibrosis: relaxin significantly inhibits angiotensin II (Ang II)-induced proliferation and migration of neonatal rat cardiac fibroblasts (CFs), thereby reducing cardiac fibrosis.
- Mechanistic pathway: relaxin exerts its antifibrotic effects by activating the NO/cGMP signaling pathway, evidenced by increased levels of NO, cGMP, and TNOS in CFs.
- Reduction of fibrotic markers: treatment with relaxin decreases the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and type I and type III collagen, which are key markers of cardiac fibrosis.
- Specific action: relaxin does not significantly affect inducible NOS (iNOS) levels, indicating its specific action through the NO/cGMP pathway.

highly selective and irreversible inhibitor of soluble guanylate cyclase (sGC), blocking NO-induced cGMP synthesis.

### Immunofluorescence

Cells cultured in 24-well plates were fixed with 4% paraformaldehyde for 30 minutes at room temperature. The cells were then rinsed 3 times with PBS, permeabilized in 0.3% Triton X-100 for 30 minutes, and rinsed 3 additional times with PBS. After blocking with 5% goat serum for 1 hour in PBS, the cells were incubated overnight at 4°C with vimentin (1 : 100),  $\alpha$ -SMA (1 : 50), goat anti-rabbit IgG (H&L), TRITC, and goat anti-mouse IgG (H&L), TRITC antibodies for 1 hour. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Negative controls were performed by omitting the primary antibodies. After washing, immunofluorescence visualization was performed using a fluorescence microscope. All results were based on independent analyses performed in triplicate.

### Cell Proliferation and Cell Migration Assays

To assess whether ODQ or L-NAME influenced the inhibitory effect of relaxin on cell proliferation, CFs were first incubated with Ang II (0.1  $\mu$ M) and then treated with relaxin (100 ng/mL) in combination with L-NAME (100  $\mu$ M) or ODQ (10  $\mu$ M) for 72 hours. Subsequently, MTT and EdU incorporation assays were performed. A transwell chamber assay was employed to evaluate the effects of relaxin on CF migration.

Briefly, CFs were seeded into 96-well plates at a density of  $1 \times 10^4$  cells/mL with 100  $\mu$ L of cell suspension per well and allowed to adhere in DMEM containing 10% FBS for 24 hours. They were serum-starved overnight and then treated with Ang II (0.1  $\mu$ M) alone or in combination with ODQ (10  $\mu$ M) or L-NAME (100  $\mu$ M), with or without relaxin (100 ng/mL), in 1% FBS DMEM for 72 hours. After 4 hours of incubation, the reaction was halted by adding 10  $\mu$ L of MTT (5 mg/mL). The supernatant was removed, and 150  $\mu$ L of DMSO was added to each well for 10 minutes. Finally, absorbance was measured at 490 nm using a microplate spectrophotometer. The assay was performed in triplicate.

### Proliferation Assay

For the EdU assay, CFs ( $1 \times 10^5$  cells/mL) were seeded into 24-well plates and treated with Ang II (0.1  $\mu$ M) alone or in combination with ODQ (10  $\mu$ M) or L-NAME (100  $\mu$ M), with or without relaxin (100 ng/mL), in 1% FBS DMEM for 72 hours. The cells were then incubated with 50  $\mu$ mol/L EdU for an additional 2 hours in a 5% CO<sub>2</sub> incubator at 37°C. Subsequently, cells were fixed with 4% paraformaldehyde for 30 minutes and decolorized with glycine (2 mg/mL), followed by a PBS wash and permeabilization with 0.5% Triton X-100 for 10 minutes at room temperature. After a PBS wash, CFs were incubated with 200  $\mu$ L of 1 $\times$ Apollo reaction cocktail for 30 minutes, followed by treatment with 0.5% Triton X-100 for 10 minutes, twice. The cells were then washed twice with methanol for 5 minutes each and once with PBS. The DNA content of the cells was stained with 100  $\mu$ L of Hoechst 33342 (5  $\mu$ g/mL) for 30 minutes and visualized using a fluorescence microscope.

### Migration Assays

Migration assays were performed using 24-well cell culture plates with polyethylene terephthalate membranes (8.0  $\mu$ m pore size, 10 mm diameter, Corning). Cardiac fibroblasts were seeded in the upper transwell chambers at a density of  $1 \times 10^5$  cells per well. Angiotensin II (0.1  $\mu$ M), either alone or combined with ODQ (10  $\mu$ M) or L-NAME (100  $\mu$ M), with or without relaxin (100 ng/mL), was added to the lower transwell chambers containing 700  $\mu$ L of DMEM with 1% FBS. After 24 hours of incubation in 5% CO<sub>2</sub> at 37°C, non-migrating cells in the upper chambers were removed with cotton swabs, while cells in the lower chambers were fixed with 4% paraformaldehyde for 30 minutes, followed by crystal violet staining for 30 minutes. Subsequently, the number of migrated cells was counted under  $\times 100$  magnification using a light microscope, with 5 randomly selected fields of view per well. The control CF migration was set as 100%, and the results were expressed as relative migration rates.

### Western Blot Analysis

Cardiac fibroblasts were collected after 72 hours of pretreatment with Ang II, or a combination of Ang II with ODQ or L-NAME, with or without relaxin. The cells were washed twice with PBS and then mixed with cell lysis buffer. The cell-buffer mixture was then scraped from the plate and kept on ice for 15 minutes. The lysates were centrifuged at 12000 g for 15 minutes at 4°C. The supernatants were collected, and protein concentrations were determined using a BCA protein assay kit, with bovine serum albumin as the standard. Subsequently, sodium dodecyl sulfate (SDS) was applied to denature the proteins. The cell extracts were separated using electrophoresis on an 8% SDS-polyacrylamide gel. After transferring to a polyvinylidene fluoride membrane, alkaline phosphatase-conjugated rabbit anti-goat, goat anti-rabbit, and anti-mouse IgG antibodies were applied for type I collagen (1 : 250), type III collagen (1 : 250),  $\alpha$ -SMA (1 : 250), and  $\beta$ -actin (1 : 5000), respectively. The results were photographed after 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue-tetrazolium staining. The intensity of the type I collagen, type III collagen, and  $\alpha$ -SMA bands was analyzed using the ImageJ 3.0 software for densitometric analysis. Finally, the protein band ratios were quantified by comparing them to  $\beta$ -actin bands from the same sample.

### Nitric Oxide Level Measurement

Nitric oxide levels were measured from culture media samples pretreated with Ang II, Ang II combined with ODQ, or L-NAME, with or without relaxin, for 72 hours. Nitrite levels in the cell-free medium were considered a reflection of NO production and were used to assess NO levels by employing the Griess reagent. Nitrite concentrations in the samples were calculated using a sodium nitrite standard curve. Absorbance was measured at 550 nm.

### Measurement of Total NOS and iNOS Levels

Total nitric oxide synthase (TNOS) activity was quantified using a colorimetric assay kit according to the manufacturer's instructions. The assay detects the enzymatic conversion of substrates into NO, with NO quantified as the reaction product. The absorbance of the final reaction product was measured at 530 nm to determine the TNOS activity.

Inducible nitric oxide synthase (iNOS) levels were quantified using a specific ELISA kit. The culture medium was collected and processed following the manufacturer's instructions. The assay relies on the binding of anti-iNOS antibodies to iNOS present in the samples. A secondary antibody conjugated with horseradish peroxidase (HRP) was applied, followed by a colorimetric reaction. Absorbance was measured at 540 nm, and iNOS levels were determined by comparison with a standard curve generated from known iNOS concentrations.

### Measurement of cGMP Concentration in Culture Media Samples

The concentration of cGMP in CF culture media, representing NO bioavailability, was measured after 72 hours of pretreatment with Ang II, Ang II combined with ODQ or L-NAME, with or without relaxin, using rat cGMP ELISA kits according to the manufacturer's instructions. The assay employed a double-antibody sandwich ABC-ELISA method. Standards and sample cGMP were conjugated with anti-rat monoclonal antibodies and coated onto microplates, followed by the addition of biotinylated anti-rat cGMP to form immune complexes, and further conjugation with horseradish peroxidase-labeled streptavidin. A blue substrate working solution was then added to the plates, followed by a sulfuric acid stop solution, after which Optical Density (OD) values were measured at 450 nm. The cGMP concentration in samples was proportional to the OD intensity at 450 nm and was determined using a standard curve generated from provided cGMP standards.

### Statistical Analysis

All data were expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software. Before any comparisons, a Shapiro–Wilk test was conducted to assess the distribution of the data. If the data were normally distributed, comparisons between multiple groups were conducted using one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons. Conversely, if the data were not normally distributed, the Kruskal–Wallis *H* test was applied, followed by Dunn's post hoc test for pairwise comparisons. A *P*-value of  $<.05$  was considered statistically significant. All experiments were performed in triplicate.

## RESULTS

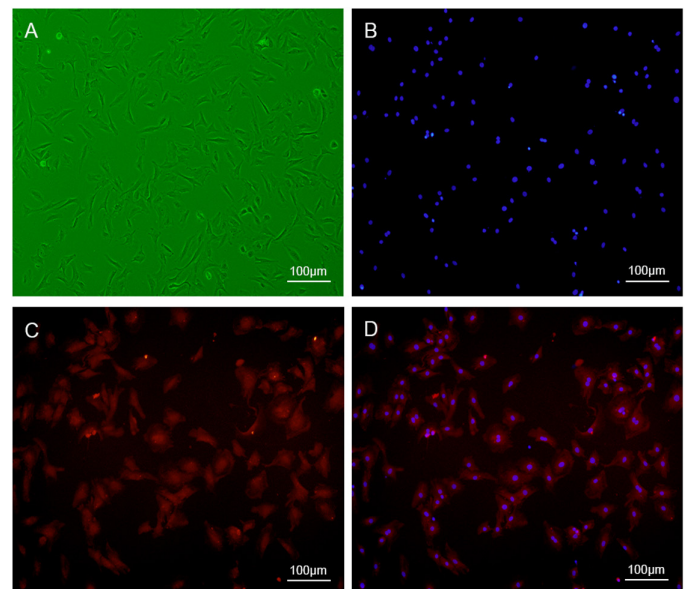
### Identification of Cultured Cardiac Fibroblasts

The cultured CFs displayed characteristic long fusiform or polygonal morphology with radial or volute-shaped aggregate growth, as observed under the microscope (Figure 1A). The nuclei were prominently stained with DAPI, showing clear nucleoli, and in some cells, 2–3 nucleoli were visible (Figure 1B). Immunofluorescence staining revealed that 99% of the cultured cells expressed vimentin, a marker of mesenchymal cells, confirming their fibroblast identity (Figure 1C). Notably,  $\alpha$ -SMA expression was absent, indicating that the CFs had not undergone activation to myofibroblasts (Figure 1D). These results confirm that the majority of the cultured cells were non-activated CFs.

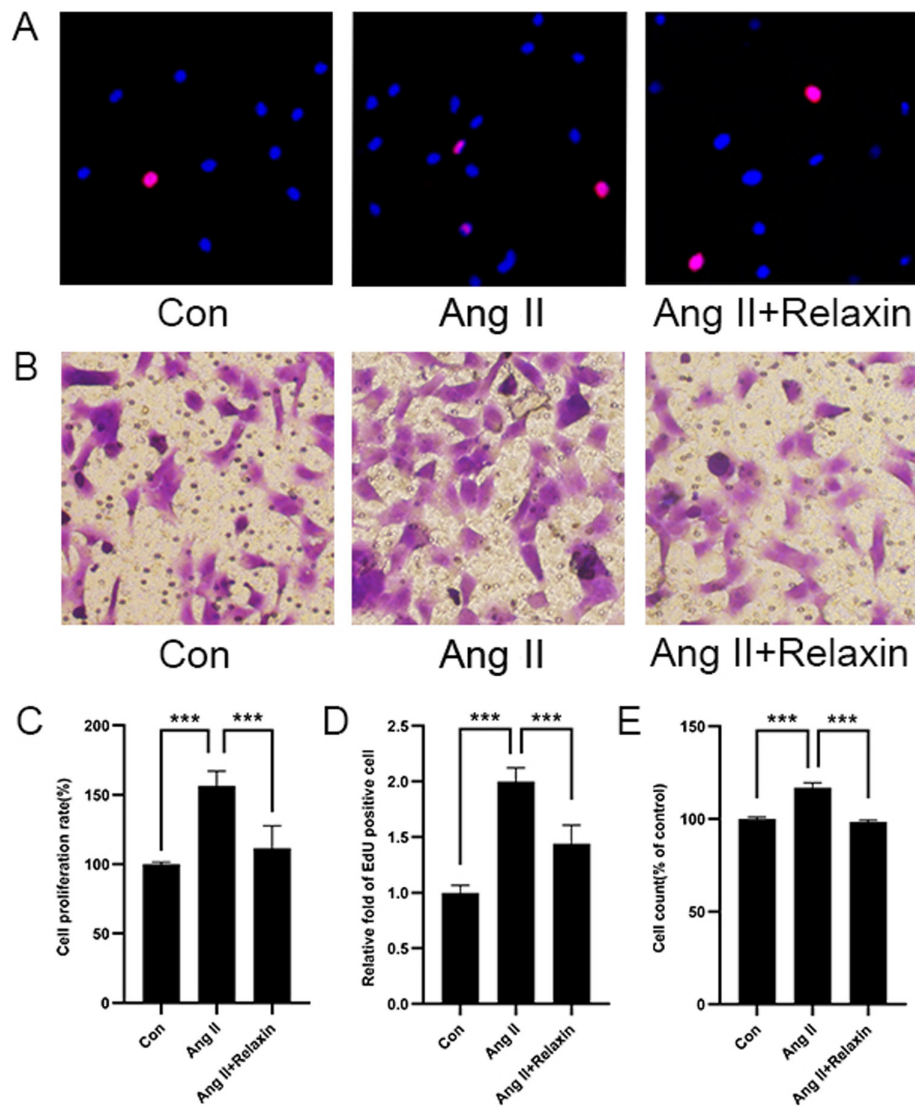
### Effects of Relaxin on Angiotensin II-Induced Cardiac Fibroblast Proliferation and Migration

As shown in Figure 2, Ang II treatment significantly increased CF proliferation and migration compared to the control group. 5-Ethynyl-2'-deoxyuridine staining revealed an increase in EdU-positive cells (indicative of proliferation) in the Ang II-treated group, while treatment with relaxin reversed this effect, reducing the number of EdU-positive cells (Figure 2A). Similarly, crystal violet staining demonstrated that Ang II treatment led to a higher number of migrated cells compared to the control, an effect that was attenuated by relaxin (Figure 2B).

Quantitative analysis confirmed that Ang II treatment significantly increased the cell proliferation rate compared to the control ( $P < .001$ ), while relaxin significantly reduced the proliferation rate in the Ang II + relaxin group ( $P < .001$ , Figure 2C). The relative fold of EdU-positive cells was also significantly elevated in the Ang II group compared to the control ( $P < .001$ ), and relaxin treatment lowered this number ( $P < .001$ , Figure 2D). Migration assays further showed that Ang II significantly enhanced CF migration ( $P < .001$ ), and relaxin treatment reduced this migration ( $P < .001$ , Figure 2E). These findings suggest that relaxin can inhibit Ang II-induced proliferation and migration of CFs (Table 1).



**Figure 1. Identification of cultured cardiac fibroblasts (CFs).** (A) Bright-field image of cultured CFs, displaying long fusiform or polygonal morphology with radial or volute-shaped aggregate growth. (B) DAPI staining of CFs highlighting prominent nuclei with visible nucleoli. (C) Immunofluorescence staining shows positive vimentin expression in CFs, a marker of mesenchymal cells. (D) Merged image of vimentin (red) and DAPI (blue) confirms the absence of  $\alpha$ -SMA expression, indicating that the majority of the cultured cells are CFs and not activated myofibroblasts. Scale bars: 100  $\mu$ m.



**Figure 2.** Effects of relaxin on angiotensin II (Ang II)-induced CF proliferation and migration. (A) 5-Ethynyl-2'-deoxyuridine staining showing cell proliferation: nuclei stained with DAPI (blue) and EdU-positive cells (pink) under various treatments (Control, Ang II, and Ang II + Relaxin). (B) Representative images of CF migration stained with crystal violet under different treatment conditions. (C) Quantitative analysis of cell proliferation rates across different treatments. (D) Relative fold change in EdU-positive cells among the experimental groups. (E) Quantification of migrated cells expressed as a percentage of control. Ang II significantly increased proliferation, while co-treatment with relaxin reduced this effect ( $***P < .001$ ).

### Involvement of the NO/cGMP Pathway in Relaxin-Mediated Inhibition of Ang II-Induced Proliferation and Migration

As depicted in Figure 3, inhibition of the NO/cGMP pathway significantly reversed the suppressive effects of relaxin on Ang II-induced CF proliferation and migration. 5-Ethynyl-2'-deoxyuridine staining showed that Ang II combined with the NOS inhibitor L-NAME or the cGMP inhibitor ODQ resulted in higher levels of EdU-positive cells, indicating increased proliferation compared to the control group. Relaxin treatment alone reduced proliferation, but when co-administered with L-NAME or ODQ, this effect was diminished (Figure 3A). Similarly, crystal violet staining demonstrated increased cell migration in the Ang II+L-NAME and Ang II+ODQ groups, with relaxin treatment reducing

migration, but this effect was partially reversed when the NO/cGMP pathway was inhibited (Figure 3B).

Quantitative analysis confirmed that cell proliferation rates were significantly increased in the Ang II + L-NAME and Ang II + ODQ groups compared to controls ( $P < .001$ , Figure 3C). Relaxin significantly reduced these rates, but the effect was diminished when L-NAME or ODQ were added. The relative fold change of EdU-positive cells showed a similar trend, with Ang II + L-NAME and Ang II + ODQ groups exhibiting significantly higher numbers of EdU-positive cells compared to controls, and relaxin reducing this effect ( $P < .001$ , Figure 3D). Migration assays also showed increased cell migration in the presence of Ang II + L-NAME and Ang II + ODQ ( $P < .001$ ), with relaxin reducing migration, though this reduction was

**Table 1. Effects of Relaxin on Angiotensin II (Ang II)-Induced CF Proliferation and Migration**

	Con	Ang II	Ang II+Relaxin	P
Cell proliferation rate (%)	100.00 ± 1.22	156.35 ± 9.64***	111.62 ± 14.24###	<.001
Relative fold of EdU positive cell	1.00 ± 0.06	2.00 ± 0.11***	1.44 ± 0.15###	<.001
Cell count (% of control)	100.00 ± 0.96	116.86 ± 2.25***	98.48 ± 0.82###	<.001

Values were expressed as mean ± standard error of the mean (SEM), n = 5.

Ang II, angiotensin II; EdU, 5-Ethynyl-2'-deoxyuridine.

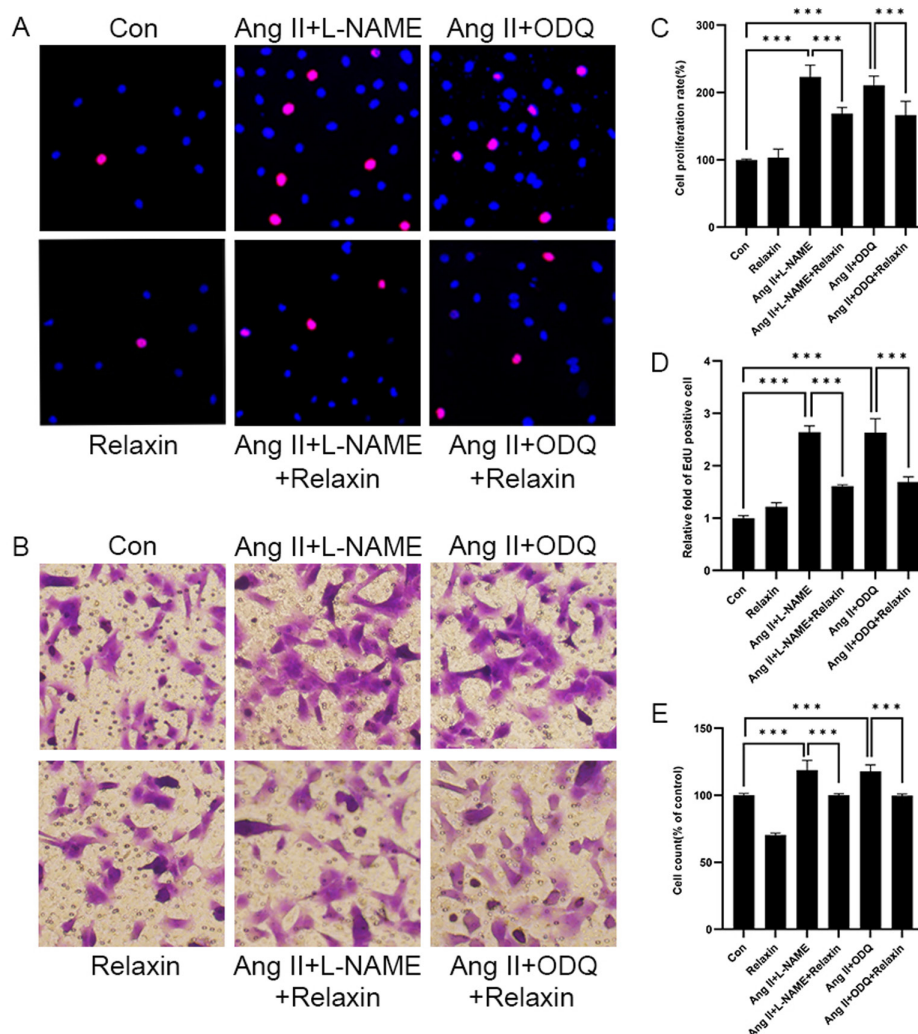
\*\*\*P < .001 vs. Con.

###P < .001 vs. Ang II.

attenuated by L-NAME or ODQ ( $P < .001$ , Figure 3E). These results demonstrate that the NO/cGMP pathway is critically involved in mediating the antifibrotic effects of relaxin on CFs (Table 2).

### Effects of Relaxin on Ang II-Induced $\alpha$ -SMA, Collagen I, and Collagen III Expression

As shown in Figure 4, treatment with Ang II significantly increased the expression of  $\alpha$ -SMA, collagen I, and collagen III in CFs, indicating enhanced fibrosis. Western blot analysis confirmed elevated levels of these fibrosis markers in the Ang II group, while treatment with relaxin effectively reduced their expression (Figure 4A). However, the co-administration of L-NAME (a NOS inhibitor) or ODQ (a cGMP inhibitor) with Ang II partially reversed the inhibitory effects of relaxin on these markers, resulting in higher levels of  $\alpha$ -SMA, collagen



**Figure 3. Involvement of NO/cGMP pathway in relaxin-mediated inhibition of Ang II-induced proliferation and migration. (A)** 5-Ethynyl-2'-deoxyuridine staining of CFs across different groups: Con, Relaxin, Ang II + L-NAME (NOS inhibitor), Ang II + ODQ (cGMP inhibitor), and combinations of Relaxin with L-NAME or ODQ. **(B)** Representative images of CF migration stained with crystal violet across different groups. **(C)** Quantitative comparison of cell proliferation rates. **(D)** Relative fold change in EdU-positive cells across groups. **(E)** Quantitative analysis of migrated cells as a percentage of the control group. \*\*\* $P < .001$ . Inhibition of the NO/cGMP pathway with L-NAME or ODQ diminished relaxin's ability to inhibit Ang II-induced proliferation (\*\*\* $P < .001$ ).

**Table 2. Involvement of NO/cGMP Pathway in Relaxin-Mediated Inhibition of Ang II-induced Proliferation and Migration**

	Con	Relaxin	Ang II + L-NAME	Ang II + L-NAME + Relaxin	Ang II + ODQ	Ang II + ODQ + Relaxin	P
Cell proliferation rate (%)	100.00 ± 1.13	103.33 ± 10.33	223.11 ± 14.36	168.53 ± 7.69	210.72 ± 11.24	166.46 ± 16.69	<.001
Relative fold of EdU positive cell	1.00 ± 0.04	1.22 ± 0.06	2.64 ± 0.10	1.61 ± 0.02	2.63 ± 0.22	1.69 ± 0.08	<.001
Cell count (% of control)	100.00 ± 0.98	70.45 ± 0.96	118.83 ± 5.93	100.02 ± 0.93	117.81 ± 3.97	99.86 ± 0.92	<.001

Values were expressed as mean ± standard error of the mean (SEM), n = 3.

Ang II, angiotensin II; EdU, 5-Ethynyl-2'-deoxyuridine; L-NAME, NG-nitro-L-arginine methyl ester; ODQ, Oxadiazolo-(4,3-a) quinoxalin-1-one.

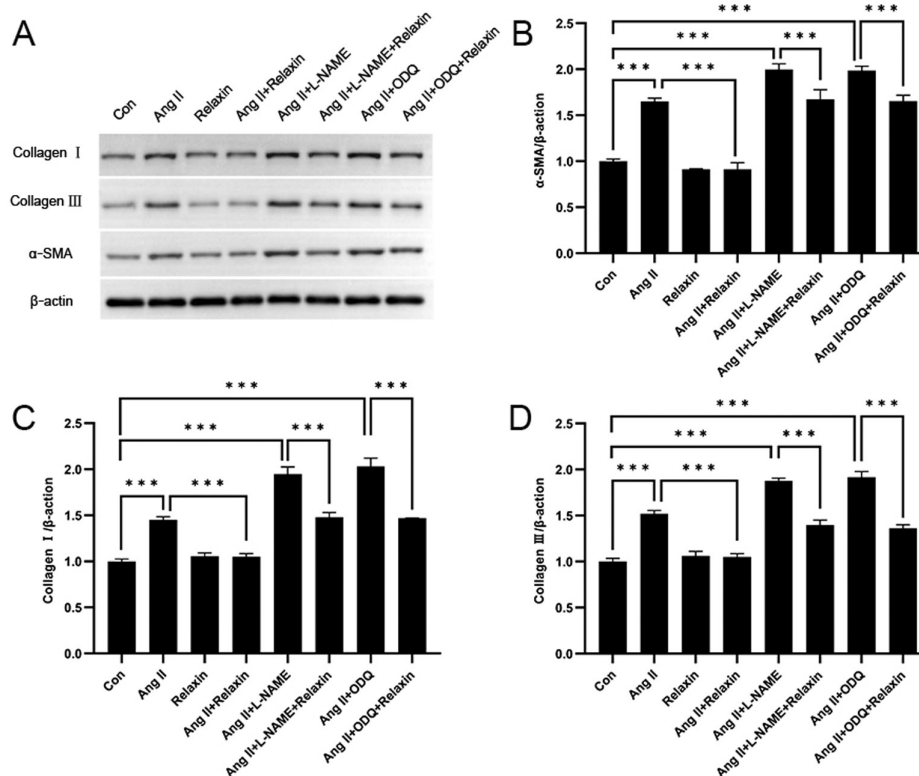
I, and collagen III in the Ang II + L-NAME + Relaxin and Ang II + ODQ + Relaxin groups compared to the Ang II + Relaxin group.

Quantitative analysis revealed that Ang II significantly increased  $\alpha$ -SMA expression compared to the control group ( $P < .001$ , Figure 4B), while relaxin treatment reduced this increase. However, in the presence of L-NAME or ODQ, relaxin's inhibitory effect was diminished ( $P < .001$ ). Similar trends were observed for collagen I and collagen III expression, where Ang II significantly upregulated these proteins ( $P < .001$ , Figure 4C-D), and relaxin reduced their levels, but this reduction was partially reversed by L-NAME or ODQ ( $P < .001$ ). These findings suggest that the NO/cGMP pathway

plays a critical role in relaxin's ability to inhibit Ang II-induced fibrosis in CFs (Table 3).

### Nitric Oxide/Cyclic Guanosine Monophosphate Signaling Pathway Activation by Relaxin in CFs

As shown in Figure 5, treatment with Ang II significantly reduced NO and cGMP levels in the culture medium. Relaxin treatment restored both NO and cGMP levels, but this effect was attenuated when the NO/cGMP pathway was inhibited by L-NAME or ODQ. Specifically, Ang II reduced NO levels compared to the control group ( $P < .001$ ), and relaxin treatment restored NO levels. However, co-treatment with L-NAME or ODQ partially reversed relaxin's effect ( $P < .001$ , Figure 5A). Similarly, Ang II significantly decreased cGMP



**Figure 4. Effects of relaxin on Ang II-induced  $\alpha$ -SMA, collagen I, and collagen III expression. (A) Western blot showing the expression of  $\alpha$ -SMA, collagen I, and collagen III across treatment groups: Con, Ang II, Relaxin, Ang II + Relaxin, Ang II + L-NAME, Ang II + L-NAME + Relaxin, Ang II + ODQ, and Ang II + ODQ + Relaxin. (B-D) Quantitative analysis of  $\alpha$ -SMA (B), Collagen I (C), and Collagen III (D) normalized to  $\beta$ -actin. Angiotensin II significantly increased the expression of these markers, which was reduced by Relaxin. However, L-NAME or ODQ attenuated relaxin's inhibition of these fibrosis markers (\*\*\*)  $P < .001$ .**

**Table 3. Effects of Relaxin on Ang II-Induced  $\alpha$ -SMA, Collagen I, and Collagen III Expression**

	Con	Ang II	Relaxin	Ang II + Relaxin	Ang II + L-NAME	Ang II + L-NAME + Relaxin	Ang II + ODQ	Ang II + ODQ + Relaxin	P
$\alpha$ -SMA/ $\beta$ -action	1.00 $\pm$ 0.02	1.65 $\pm$ 0.03	0.91 $\pm$ 0.01	0.91 $\pm$ 0.07	1.99 $\pm$ 0.06	1.67 $\pm$ 0.10	1.98 $\pm$ 0.05	1.65 $\pm$ 0.06	<.001
Collagen I/ $\beta$ -action	1.00 $\pm$ 0.03	1.45 $\pm$ 0.03	1.06 $\pm$ 0.04	1.05 $\pm$ 0.03	1.94 $\pm$ 0.07	1.48 $\pm$ 0.05	2.03 $\pm$ 0.09	1.47 $\pm$ 0.01	<.001
Collagen III/ $\beta$ -action	1.00 $\pm$ 0.03	1.52 $\pm$ 0.03	1.06 $\pm$ 0.05	1.05 $\pm$ 0.04	1.88 $\pm$ 0.03	1.39 $\pm$ 0.05	1.91 $\pm$ 0.06	1.36 $\pm$ 0.04	<.001

Values were expressed as mean  $\pm$  standard error of the mean (SEM), n = 3.

Ang II, angiotensin II; EdU, 5-Ethynyl-2'-deoxyuridine; L-NAME, NG-nitro-L-arginine methyl ester; ODQ, Oxadiazolo-(4,3-a) quinoxalin-1-one.

levels ( $P < .001$ ), which were restored by relaxin, though this effect was diminished in the presence of L-NAME or ODQ ( $P < .05$ , Figure 5B).

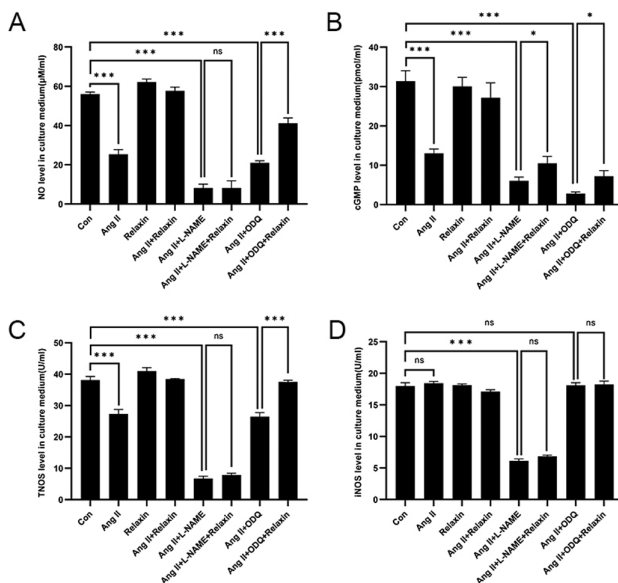
Total NOS levels were also reduced by Ang II and restored by relaxin. However, L-NAME treatment significantly blocked the effect of relaxin on TNOS levels ( $P < .001$ , Figure 5C). Interestingly, iNOS levels remained largely unchanged across groups, except for a significant reduction in the L-NAME-treated group ( $P < .001$ , Figure 5D). These findings confirm that relaxin mediates its antifibrotic effects through the NO/cGMP signaling pathway, primarily by restoring NO and cGMP levels, while TNOSTNOS appears to play a more central role than iNOS in this process (Table 4).

## DISCUSSION

In this study, we explored the effects of relaxin on Ang II-induced CF activation and fibrosis, with a particular focus on the role of the NO/cGMP signaling pathway. We demonstrated that relaxin markedly reduces CF proliferation, migration, and the expression of fibrosis markers, such as  $\alpha$ -SMA, collagen I, and collagen III. Furthermore, our results show that relaxin's antifibrotic effects are closely tied to the NO/cGMP pathway, as blocking this pathway with L-NAME or ODQ partially reversed its effects. Additionally, we found that relaxin restored NO, cGMP, and TNOS levels reduced by Ang II, highlighting the central role of NO signaling in relaxin's mechanism of action. Interestingly, while TNOS levels were significantly modulated by relaxin, iNOS levels were largely unaffected, except in the presence of L-NAME, which showed a significant reduction. These findings underscore the therapeutic potential of relaxin in cardiac fibrosis and emphasize the importance of the NO/cGMP pathway.

Our findings align with previous studies that have demonstrated relaxin's antifibrotic effects in various models of cardiovascular disease. Wilhelmi et al<sup>11</sup> showed that relaxin, particularly its recombinant form serelaxin, significantly reduces cardiac fibrosis by inhibiting fibroblast-to-myofibroblast transition and reducing collagen production. This study further demonstrated that the effects of relaxin are mediated through its receptor RXFP1 and the NO/cGMP pathway. Similarly, Wang et al<sup>25</sup> found that relaxin exerted antifibrotic effects in high-salt-fed mice by enhancing NO production and cGMP levels, preventing fibroblast activation. Our results corroborate these findings, particularly the observation that relaxin can reverse the pro-fibrotic effects of Ang II through the modulation of NO production and the downstream cGMP signaling cascade.

The NO/cGMP pathway is a well-established regulator of fibroblast activation and cardiac fibrosis.<sup>26,27</sup> Nitric oxide, produced by various isoforms of NOS, plays a key role in modulating fibrosis by inhibiting fibroblast proliferation and collagen deposition.<sup>28</sup> In our study, Ang II significantly reduced NO and cGMP levels, consistent with the known effects of Ang II in promoting fibrosis by inhibiting protective NO signaling. Relaxin treatment restored NO and cGMP levels, and this effect was diminished in the presence of L-NAME or ODQ, confirming the involvement of the NO/cGMP pathway in relaxin's antifibrotic actions. This is consistent with



**Figure 5. NO/cGMP signaling pathway activation by relaxin in CFs. (A) NO levels in the culture medium. Ang II reduced NO levels, and relaxin restored them, though this effect was diminished by L-NAME and ODQ (\*\*\*) ( $P < .001$ ). (B) cGMP levels were similarly reduced by Ang II and restored by relaxin, with L-NAME and ODQ partially inhibiting this effect ( $*P < .05$ ,  $***P < .001$ ). (C) Total NOS (TNOS) levels were reduced by Ang II and restored by relaxin, though L-NAME blocked relaxin's effect ( $***P < .001$ ). (D) iNOS levels remained largely unchanged across groups, except for a reduction in the L-NAME-treated group ( $***P < .001$ ).**



**Table 4. Nitric Oxide/Cyclic Guanosine Monophosphate Signaling Pathway Activation by Relaxin in CFs**

	Con	Ang II	Relaxin	Ang II + Relaxin	Ang II + L-NAME	Ang II + L-NAME + Relaxin	Ang II + ODQ	Ang II + ODQ + Relaxin	P
NO level in culture medium ( $\mu$ M/mL)	56.01 $\pm$ 0.91	25.43 $\pm$ 1.91	62.15 $\pm$ 1.28	57.73 $\pm$ 1.58	8.25 $\pm$ 1.58	8.25 $\pm$ 2.98	20.96 $\pm$ 0.91	41.24 $\pm$ 2.15	<.001
cGMP level in culture medium (pmol/mL)	31.38 $\pm$ 2.19	13.00 $\pm$ 0.95	30.05 $\pm$ 1.89	27.14 $\pm$ 3.10	6.06 $\pm$ 0.79	10.48 $\pm$ 1.45	2.82 $\pm$ 0.33	7.23 $\pm$ 1.15	<.001
TNOS level in culture medium (U/mL)	38.16 $\pm$ 0.91	27.34 $\pm$ 1.14	40.97 $\pm$ 0.93	38.45 $\pm$ 0.15	6.73 $\pm$ 0.64	7.90 $\pm$ 0.44	26.46 $\pm$ 1.02	37.58 $\pm$ 0.39	<.001
iNOS level in culture medium (U/mL)	17.98 $\pm$ 0.44	18.42 $\pm$ 0.25	18.13 $\pm$ 0.15	17.11 $\pm$ 0.25	6.14 $\pm$ 0.25	6.87 $\pm$ 0.15	18.09 $\pm$ 0.41	18.23 $\pm$ 0.54	<.001

Values were expressed as mean  $\pm$  standard error of the mean (SEM), n = 3.

Ang II, angiotensin II; EdU, 5-Ethynyl-2'-deoxyuridine; L-NAME, NG-nitro-L-arginine methyl ester; ODQ, Oxadiazolo-(4,3-a) quinoxalin-1-one.

previous studies, including those by Tapia Cáceres et al,<sup>18</sup> which demonstrated that relaxin inhibits fibroblast activation through NO-dependent mechanisms.

Interestingly, our study also revealed that iNOS levels were largely unaffected by relaxin, except for a significant reduction in the L-NAME group. This suggests that the NO production responsible for relaxin's antifibrotic effects may be primarily derived from endothelial nitric oxide synthase (eNOS) or neuronal nitric oxide synthase (nNOS) rather than iNOS. Inducible nitric oxide synthase is typically associated with inflammation and the production of large amounts of NO during inflammatory responses, which can contribute to oxidative stress and tissue damage.<sup>29</sup> The lack of significant changes in iNOS levels in our study implies that fibrosis induced by Ang II in CFs may not be driven by an inflammatory mechanism, and that relaxin's effects are more likely related to the regulation of protective NO signaling pathways via eNOS or nNOS. This is consistent with findings by Chow et al,<sup>30</sup> who demonstrated that relaxin primarily modulates NO production through eNOS and that this mechanism is crucial for its cardiovascular protective effects.

In our experiment, the lack of significant changes in iNOS levels, except for the reduction in the L-NAME group, indicates that iNOS may not play a major role in Ang II-induced fibrosis in CFs. It is possible that other factors, such as eNOS or transforming growth factor beta (TGF- $\beta$ ) signaling, may be more important in this context, and further studies are needed to clarify the specific contributions of different NOS isoforms to the fibrotic process in CFs.

While this study offers important insights into relaxin's role in cardiac fibrosis via the NO/cGMP pathway, some limitations must be noted. First, the use of isolated CFs in vitro may not fully capture the complexity of cardiac fibrosis in vivo. Future studies in animal models or clinical samples are needed to validate these findings. Second, while we focused on the NO/cGMP pathway, other pathways like PI3K/Akt and TGF- $\beta$  may also contribute to relaxin's effects and were not explored here. Lastly, iNOS levels were largely unaffected in

our study, and its role in cardiac fibrosis, particularly under inflammatory conditions, remains unclear. Further research is needed to better understand iNOS's involvement and its potential interaction with relaxin.

## CONCLUSION

This study demonstrates that relaxin plays a key role in inhibiting Ang II-induced cardiac fibrosis through the activation of the NO/cGMP signaling pathway. By restoring NO and cGMP levels, relaxin reduces fibroblast activation, collagen synthesis, and migration. The involvement of the NO/cGMP pathway was confirmed by using inhibitors like L-NAME and ODQ, indicating its essential role in relaxin's antifibrotic effects. While iNOS levels remained relatively unchanged, the role of eNOS and nNOS may be more prominent in this context. Future research should focus on validating these findings in animal models or clinical settings, as well as exploring other potential pathways involved in relaxin's antifibrotic actions, such as PI3K/Akt and TGF- $\beta$ . Expanding this understanding could lead to the development of novel therapies for cardiac fibrosis and related heart conditions.

**Animals and Reagents:** Neonatal SD rats were obtained from the animal center of Xuzhou Medical University. Recombinant human relaxin-2 (relaxin) was obtained from the Connetics Corporation (Palo Alto, CA). Ang II, MTT, L-NAME, ODQ, and DMSO were purchased from Sigma-Aldrich. 5-ethynyl-20-deoxyuridine was obtained from Ribobio Biotechnology. Type I and type III collagen and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibodies were purchased from Santa Cruz Biotechnology Inc. Vimentin, goat anti-rabbit IgG (H&L), TRITC antibody, and BCA protein assay kit were purchased from Bioworld Inc. Enzyme-linked immunosorbent assay kits for cGMP were purchased from Western Tang. Dulbecco's modified Eagle's medium, FBS, and 0.25% trypsin were purchased from Gibco BRL. Nitric oxide and NOS reagent kits were purchased from Jiancheng Bioengineering Institute. Analytical grade reagents were used in the study.

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