Relaxin Reverses Cardiac and Renal Fibrosis in Spontaneously Hypertensive Rats

Edna D. Lekgabe, Helen Kiriazis, Chongxin Zhao, Qi Xu, Xiao Lei Moore, Yidan Su, Ross A.D. Bathgate, Xiao-Jun Du, Chrishan S. Samuel

Abstract—The antifibrotic effects of the peptide hormone relaxin on cardiac and renal fibrosis were studied in 9- to 10-month-old male spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). Rats (n=8 to 9 per group) were allocated into 3 groups: WKY controls, vehicle-treated SHR (SHR-V), and relaxin-treated SHR (SHR-R). Relaxin (0.5 mg/kg per day) was administered via subcutaneously implanted osmotic mini-pumps over 2 weeks before hearts and kidneys were harvested for analysis. Collagen content was analyzed by hydroxyproline assay, gel electrophoresis, and quantitative histology. Zymography was used to determine matrix metalloproteinase (MMP) expression and Western blotting to determine proliferating cell nuclear antigen (PCNA) expression, whereas cardiac hypertrophy was assessed by myocyte size and real-time polymerase chain reaction of associated genes. The left ventricular (LV) myocardium of SHR-V contained increased collagen levels (by 25±1%, P<0.01 using biochemical analysis and 3-fold; P<0.01 using quantitative histology), enhanced expression of PCNA (by 70±8%; P<0.01), α-SMA (by 32±2%; P<0.05), and the collagen-degrading enzyme MMP-9 (by 70±6%; P<0.05) versus respective levels measured in WKY controls. The kidneys of SHR-V also contained increased collagen (25±2%, P<0.05 using biochemical analysis and 2.4-fold; P<0.01 using quantitative histology). Relaxin treatment significantly normalized collagen content in the LV (P<0.01) and kidney (P<0.05), completely inhibited cell proliferation (P<0.01) and fibroblast differentiation (P<0.05) in the LV, and increased MMP-2 expression (by 25±1%; P<0.05) without affecting MMP-9 in the LV compared with that measured in SHR-V. Thus, relaxin is a potent antifibrotic hormone with a rapid-occurring efficacy that may have therapeutic potential for hypertensive disease. (Hypertension. 2005;46:412-418.)

Key Words: rats ■ extracellular matrix ■ remodeling ■ fibrosis

Hypertension affects 15% to 20% of the adult population, leading to structural remodeling of the left ventricular (LV) myocardium and eventually heart failure. Several studies have focused on the contribution of the extracellular matrix (ECM) to cardiac diastole and systole function. It is now well established that the fibrillar collagen network in the ECM is integral in providing the structural support for cardiomyocytes and coronary vessels. It imparts the myocardium with physical properties and influences ventricular diastolic and systolic function. A pathological stimulus to this network leads to the development of cardiac fibrosis, an integral characteristic of hypertensive heart disease.

Cardiac fibrosis is a hallmark of hypertensive heart disease and interferes with normal structure and function of the myocardium. Cardiac fibroblasts are activated and differentiate into myofibroblasts after cardiomyocyte death, inflammation, enhanced workload, hypertrophy, and stimulation by a number of hormones (eg, angiotensin II), cytokines (eg, interleukin-1), and growth factors (eg, transforming growth factor-β [TGF-β]). Studies in hypertensive patients have demonstrated focal increases in fibrillar collagen types I and III, as well as a more diffuse deposition of basement membrane collagen type IV in the LV. It is this ECM remodeling that raises tissue stiffness, predisposing patients with hypertensive heart disease to increased risk of adverse cardiac events, including myocardial ischemia and infarction, diastolic or systolic ventricular dysfunction, arrhythmias, and sudden cardiac death. Thus, the reversal of cardiac fibrosis is essential in the management of hypertensive heart disease, and hence, effective treatment strategies need to be investigated.

Renal fibrosis is another major complication associated with the development and progression of hypertension. Abnormal accumulation of collagens around renal resistance vessels, glomeruli, and interstitium contributes to remodeling of vasculatures and progression of renal injury. Reversal of the renal fibrotic process associated with hypertension is expected to halt the progression of hypertension and renal failure.
Relaxin, a 6-kDa polypeptide hormone, has been shown to have several functions in mammals, including an ability to remodel the ECM of the reproductive organs, skin, lung, liver, kidney, and, more recently, the heart. It acts by reducing collagen synthesis, increasing expression of matrix metalloproteinases (MMPs) to degrade collagen, and antagonizing the influence of profibrotic factors. We showed that relaxin-deficient mice experience LV diastolic dysfunction because of elevated collagen content and underwent age-related renal fibrosis, leading to altered kidney structure and function. In separate studies, relaxin administration in vitro was able to decrease collagen deposition, fibroblast proliferation, and differentiation in rat cardiac fibroblasts stimulated with either angiotensin II or TGF-β and was also able to downregulate rat renal fibroblast function and matrix remodeling. Furthermore, relaxin administration in vivo reduced collagen content in 2 murine models of established fibrotic cardiomyopathy and reversed renal fibrosis caused by relaxin deficiency. These studies have identified relaxin as a naturally occurring regulator of collagen deposition.

In this study, we investigated the effects of relaxin administration on the spontaneously hypertensive rat (SHR) model with hypertension-induced cardiac and renal fibrosis and cardiac hypertrophy. Based on previous findings, we hypothesized that relaxin would reverse cardiac and renal fibrosis as a result of hypertension heart disease. The results from this study indicate that relaxin is able to potently and specifically inhibit cardiac and renal fibrosis in this model.

**Methods**

**Animals, Relaxin, and Osmotic Mini-Pump Implantation**

Nine- to 10-month-old male SHR and normotensive Wistar-Kyoto rats (WKY) used in this study were housed and maintained under standard conditions. These experiments were approved by a local animal ethics committee, which adheres to the Australian code of practice for the care and use of laboratory animals for scientific purposes. The recombinant human gene-2 (H2) relaxin was kindly provided by BAS Medical Inc. (San Mateo, Calif) and has been shown previously to be bioactive in rodents.

Animals were separated into 3 groups: untreated WKY (n=9), vehicle-treated SHR (SHR-V; n=8) and H2 relaxin-treated SHR (SHR-R; n=8). As described previously, rats underwent surgery to subcutaneously implant 14-day ALZET osmotic mini-pumps (model 2ML2; Durect Corporation), loaded either with vehicle (20 mmol/L sodium acetate buffer, pH 5.0; SHR-V) or with 0.5 mg/kg per day H2 relaxin (SHR-R). Fourteen-day pumps were used to account for the fact that rodents mount antibody responses to exogenous H2 relaxin by ∼10 days, resulting in increased and variable circulating serum levels. This dose of H2 relaxin was used previously to successfully treat several in vivo rodent models of fibrosis and to produce mean serum relaxin levels of 19 ng/mL, which is within levels found in pregnant rats (ie, 50 to 200 ng/mL).

**Cardiac Function Assessment and Tissue Collection**

Rats were anesthetized with intraperitoneal administration of ketamine/xylazine/atropine, at 60/12/0.6 mg/kg, respectively. Using SONOS 5500 ultrasound machine and a 12-MHz probe, we performed echocardiography to assess LV systolic and diastolic function immediately before and after the 2-week (H2 relaxin) treatment period, as described previously. Arterial blood and LV pressures were also assessed by catherization using a 2Fr Millar catheter immediately after final echocardiography while rats were still anesthetized, as described previously. At the end of cardiac functional assessment, hearts and kidneys were harvested for further analysis.

**Determination of Collagen Content and Subtypes**

Total collagen content in the LV myocardium and kidney cortex was determined by quantitative histology and hydroxyproline analysis. Interstitial collagen content in the LV and kidney was determined by measuring the quantity of picrosirius red and Masson trichrome staining, respectively, as a percentage of the total area within a field. Myocyte cross-sectional area was also obtained from the average of 70 to 100 cells from randomly selected fields in the LV. The hydroxyproline content of the LV, right ventricle (RV), atria, and kidneys was determined as described previously and then converted to collagen content by multiplying by a factor of 6.94. Results were then expressed as collagen concentration by dividing the collagen content by the tissue dry weight.

The LV myocardium was also finely diced and the pepsin-digested maturely cross-linked collagen obtained to determine collagen types by SDS-PAGE as described previously. An equal quantity of protein was loaded in each lane.

**Determination of Cardiac Fibroblast Function and MMP Activity**

Cell proliferation and fibroblast differentiation within the LV was examined by Western blot analysis, using monoclonal antibodies to the proliferating cell nuclear antigen (PCNA; Dako Corporation; kindly provided by Dr Tim Hewitson, Nephropathy, RMH, Victoria, Australia) and α-smooth muscle actin (α-SMA; Dako Corporation), as described previously. Western blotting of the housekeeping protein β-tubulin (monoclonal antibody kindly provided by Dr Zhonglin Chai, Baker HRI, Victoria, Australia) was also performed to demonstrate equal loading of the protein samples.

Gelatin zymography was performed to determine the effect of relaxin on MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) expression. MMPs were extracted from LV tissue and analyzed on zymogram gels as described previously.

**Real-Time Polymerase Chain Reaction Analysis of Atrial Naturetic and β-Myosin Heavy Chain Gene Expression**

Total RNA was extracted from homogenized frozen LV with the use of Trizol Reagent (Invitrogen) as described by the manufacturer. After treatment of RNase-free DNase (Promega), 1 μg of total RNA was reverse transcribed to cDNA with the use of random primers (Promega) and Superscript III RNase H Reverse Transcriptase (Invitrogen). Expression of atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC), normalized by 18S RNA, were determined by SYBR Green reactions using SYBR Green PCR (polymerase chain reaction) Master Mix and specific primers with the ABI PRISM 7700 Sequence Detection System.

**Statistical Analysis**

The echocardiographic data were analyzed using a 2-way ANOVA (to account for the 2 variables: time points and groups used), whereas all other results were analyzed using 1-way ANOVA and the Student Newman–Keuls tests for multiple comparisons between groups. All data are expressed as the mean±SEM, with P<0.05 described as statistically significant.

**Results**

**Effect of Relaxin on Morphology and Cardiac Function**

Body weights (BWVs) were moderately but significantly lower in SHR groups compared with WKY (P<0.05; Table 1). The LV and whole heart weights and LV/BW or heart/BW ratios
showed a slight increase (by 10%) of cardiomyocyte hypertrophy (Table 1). Furthermore, measurements of myocyte size were significantly higher in SHR-V and SHR-R versus WKY (Table 1). Furthermore, measurement of myocyte size showed a slight increase (by 10%) of cardiomyocyte hypertrophy in LVs from SHR (Table 1). However, this increase was not statistically significant.

Echocardiography performed before and after the 2-week treatment period revealed no significant differences in LV dimensions, wall thickness, fractional shortening, and diastolic function between the 3 groups (Table 2). With H2 relaxin administration, there was a significant increase in heart rate (P<0.05 versus SHR-V; P<0.01 versus WKY; Table 2), hence, E-wave, A-wave, E/A ratio, and deceleration time of the E-wave were unable to be quantified because of merging of E and A waves.

Effect of Relaxin on Collagen Content and Subtypes

LV collagen content was measured by quantitative histology and hydroxyproline analysis. Relative to the WKY group, SHR-V had significantly increased LV collagen content as determined by both methods (P<0.001 and P<0.01 versus WKY, respectively; Figure 1A and 1B). H2 relaxin treatment over a 2-week period significantly reduced this buildup of collagen as detected by histology (P<0.001 versus SHR-V; Figure 1A) and hydroxyproline (P<0.01 versus SHR-V; Figure 1B). In contrast to the

<table>
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<th>Parameters Measured</th>
<th>WKY</th>
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<th>SHR-R</th>
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<tr>
<td>BW, g</td>
<td>430±7</td>
<td>401±6*</td>
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<td>HW, mg</td>
<td>1271±14</td>
<td>1333±17*</td>
<td>1366±28†</td>
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<tr>
<td>LV, mg</td>
<td>982±15</td>
<td>1052±14*</td>
<td>1077±28*</td>
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<tr>
<td>RV, mg</td>
<td>193±15</td>
<td>184±10</td>
<td>188±9</td>
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<tr>
<td>Atria, mg</td>
<td>101±7</td>
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<td>HW/BW, mg</td>
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<td>3.3±0.07†</td>
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<td>LV/BW, mg/g</td>
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<td>RV/BW, mg/g</td>
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<td>Atria/BW, mg/g</td>
<td>0.2±0.02</td>
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<tr>
<td>Myocyte size, μm²</td>
<td>284±11</td>
<td>314±14</td>
<td>302±12</td>
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HW indicates heart weight.
Results are means±SEM of 8 to 9 animals per group.
*P<0.05 vs WKY; †P<0.05 vs SHR-V; ‡P<0.01 vs WKY.

were significantly higher in SHR-V and SHR-R versus WKY (Table 1). Furthermore, measurement of myocyte size showed a slight increase (by 10%) of cardiomyocyte hypertrophy in LVs from SHR (Table 1). However, this increase was not statistically significant.

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<th>WKY</th>
<th>SHR-V</th>
<th>SHR-R</th>
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<tr>
<td>HR, bpm</td>
<td>253±8</td>
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<td>253±4</td>
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<td>8.4±0.2</td>
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<td>5.2±0.36</td>
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<td>6.0±0.32</td>
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<tr>
<td>A-wave, cm/s</td>
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<tr>
<td>E/A ratio</td>
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</tr>
<tr>
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<td>17±1</td>
<td>19±2</td>
<td>17±1</td>
</tr>
<tr>
<td>ET, ms</td>
<td>95±3</td>
<td>90±2</td>
<td>93±3</td>
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</table>
| HR indicates heart rate; LVIDd and LVIDs, LV inner dimensions at diastole and systole; WTD, wall thickness at diastole; FS, fractional shortening; DT, E-wave deceleration time; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; ET, ejection time; LVEDP, LV end-diastolic pressure; E, early filling flow wave; A, late atrial filling flow wave; SAP, systolic arterial pressure.
For echocardiography, measurements from 4 to 5 cardiac cycles were taken and averaged. For hemodynamic tests, measurements were made using 10 to 12 consecutive beats and averaged.
Results are means±SEM from 8 to 9 animals per group.
*P<0.01 vs WKY; †P<0.05; ‡P<0.01 vs SHR-V.
finding in the LV, there was no significant difference in hydroxyproline content in either the RV or atria between the 3 groups (Figure 1B).

The collagen content in the kidney was also significantly increased, relative to the WKY group, as determined by both methods (P < 0.001 and P < 0.05 versus WKY, respectively; Figure 1A and 1B). Similarly, H2 relaxin treatment over 2 weeks normalized renal collagen levels by these assays (both P < 0.05 versus SHR-V; Figure 1A and 1B.)

Interstitial types I, III, and V collagen represented the mature forms of collagen in the heart of SHR and WKY and were quantified by densitometry of the bands and identified by SDS-PAGE (Figure 1C). A significant increase in types I (P < 0.01), III (P < 0.05), and V (P < 0.05) collagen was observed in SHR-V compared with that found in the WKY LV (Figure 1C). This increase was significantly reversed with H2 relaxin administration of SHR (all P < 0.05 versus SHR-V values) over a 2-week treatment period.

Effect of Relaxin on Fibroblast Function and MMP Expression

Proliferation and differentiation of fibroblasts were analyzed by Western blotting of PCNA and α-SMA expression, respectively. Fibroblast proliferation (P < 0.01 versus WKY; Figure 2A) and differentiation (P < 0.05 versus WKY; Figure 2B) were significantly increased in SHR-V. Consistent with its ability to inhibit collagen overexpression, H2 relaxin significantly inhibited PCNA (P < 0.01 versus SHR-V; Figure 2A) and α-SMA expression (P < 0.05 versus SHR-V; Figure 2B) over 2 weeks.

MMP-2 was the major gelatinase present in the LV of WKY and SHR, whereas MMP-9 expression was also detected (Figure 2C). Compared with values from WKY, a significant increase in latent MMP-9 expression (P < 0.05 versus WKY; Figure 2C) and a trend toward increased latent MMP-2 expression were detected in the LV of SHR-V. H2 relaxin treatment of SHR further increased the expression of latent MMP-2 (P < 0.05 versus WKY; Figure 2C) while
having no significant effect on MMP-9 expression versus that measured in the SHR-V group (Figure 2C).

Effect of Relaxin on Hypertrophy-Related Gene Expression

Real-time PCR of hypertrophy-related markers demonstrated that ANP expression was 4- to 5-fold higher in the SHR-V LV ($P<0.05$ versus WKY; Figure 3). However, it was not significantly affected by relaxin treatment (Figure 3). In contrast, there were no significant differences in $\beta$-MHC expression between the 3 groups studied (Figure 3).

Discussion

To our knowledge, this is the first study investigating the antifibrotic properties of relaxin in the SHR model. Our results indicate that over a 14-day period, H2 relaxin significantly reduced the elevated collagen content in the LV and kidney, in particular, types I, III, and V collagen. Furthermore, H2 relaxin inhibited fibroblast proliferation and differentiation and induced a significant rise in MMP-2 expression, factors that most likely contributed to the reduction in
collagen of the LV. The results of this study provide further evidence to support the development of relaxin as a therapeutic agent against cardiac and renal fibrosis, abnormalities induced by hypertension.

As reported by some groups using the SHR model, diastolic and systolic dysfunction was expected in the 9- to 10-month-old animals, on the basis of increased wall thickness, chamber stiffness, and impaired relaxation. However, these alterations in cardiac function were not observed in our study. Our findings were more consistent with other reports showing that despite the presence of myocardial hypertrophy and fibrosis in SHR from 3 to 6 months of age, unequivocal cardiac dysfunction did not occur until 13 to 18 months of age. With relaxin administration, there was no change in any of the recorded echocardiographic and hemodynamic parameters except for a modest but significant increase in heart rate. Furthermore, the catheter data demonstrated a trend for decreased blood pressure after relaxin treatment.

In contrast to the inconsistency regarding heart function in this model, cardiac and renal fibrosis has been documented consistently at this age. Hypertension in this model has been associated with activated sympathoadrenergic system and elevated levels of angiotensin II and TGF-β, which are well known to directly stimulate cardiac fibroblast proliferation and differentiation into activated myofibroblasts. These myofibroblasts, which are characterized by the de novo expression of α-SMA, participate in producing copious amounts of collagen and contribute to the development of fibrosis. Hence, inhibitors that antagonize profibrotic signaling and consequently fibroblast proliferation and differentiation are desirable in the treatment of fibrosis.

Three pathways were investigated by which relaxin could be acting in the LV: fibroblast proliferation, differentiation, and expression of MMPs. In accordance with previous findings, fibroblast proliferation and differentiation was found to be elevated in the heart of SHR, whereas this expression was reduced with relaxin treatment. These findings further supported our previous in vitro observations that relaxin inhibits cardiac fibroblast function. MMP-2 has been found in previous studies to be increased by relaxin and to cleave type I collagen, fibronectin, and other nonfibrillar collagens. In accordance with those findings, relaxin was found in this study to significantly increase levels of latent MMP-2 in the LV, an effect contributing to a relaxin-induced decrease in interstitial collagen.

Previous studies have reported the presence of the primary relaxin receptor LGR7 in rat cardiac and renal fibroblasts. From combined studies on relaxin knockout mice demonstrating that relaxin is a naturally occurring regulator of collagen overexpression in the aging heart and kidney, in vitro experiments showing that relaxin regulates cardiac and renal fibroblast function and matrix production, and other animal models of cardiomyopathy and renal disease demonstrating that relaxin administration in vivo decreases interstitial fibrosis and improves organ function, we propose that the mechanism of the action of relaxin to decrease collagen overexpression in the SHR LV and kidney involves its ability to: (1) directly inhibit collagen secretion; (2) inhibit activation, proliferation, and differentiation of fibroblasts; (3) increase collagen degradation by activation MMPs and inhibition of tissue inhibitors of MMP; and (4) inhibit the actions of major profibrotic factors (angiotensin II and TGF-β) in several organs. Importantly, administration of relaxin to the SHR led to a significant decrease in collagen content specifically within the kidney cortex and LV myocardium and not in the unaffected chambers of the heart. Cardiac injury constitutes an important component of hypertensive syndrome, and renal dysfunction acts as an amplifier of the syndrome. Hence, simultaneous and rapid normalization of collagen in both these organs is achieved with the use of relaxin, findings that make relaxin a more desirable intervention to current antihypertensive therapies.

Perspectives

Several studies have reported that some antihypertensive drugs such as angiotensin-converting enzyme inhibitors (eg, captopril) and aldosterone inhibitor (eg, spironolactone) do have antifibrotic properties of varying potency. However, they have undesirable side effects and are effective only after long-term treatment periods. The rapid effect at which relaxin lowers collagen overexpression in affected organs without affecting basal collagen expression in unaffected tissues makes it a desirable therapeutic agent for the reversal of organ fibrosis. Our study demonstrates novel information that relaxin selectively and rapidly inhibits collagen overexpression in the affected myocardium and the kidney of SHR without noticeable side affects, thus being able to reduce collagen accumulation as a result of hypertension. These findings suggest that relaxin is a potent and selective antifibrotic agent that may have therapeutic potential in hypertensive disease.

Acknowledgments

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References


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