Serelaxin Is a More Efficacious Antifibrotic Than Enalapril in an Experimental Model of Heart Disease

Chrishan S. Samuel, Hasangika Bodaragama, Jacqueline Y. Chew, Robert E. Widdop, Simon G. Royce, Tim D. Hewitson

Abstract—Relaxin is a naturally occurring peptide hormone that mediates systemic hemodynamic and renal adaptive changes during pregnancy and abrogates aberrant scar tissue formation (fibrosis) in diverse pathogeneses. However, its efficacy relative to renin–angiotensin system blockade, the most effective antifibrotic strategy currently available, is not known. We compared the individual versus combined antifibrotic effects of serelaxin (a recombinant form of human gene-2 relaxin) and the angiotensin-converting enzyme inhibitor enalapril, in preventative (started before injury) and therapeutic (treatment of established fibrosis) strategies, in a mouse model of isoprenaline-induced cardiac injury (at 17 days). Changes in systolic blood pressure, organ hypertrophy, and tissue remodeling/fibrosis were assessed. Pretreatment with serelaxin (0.5 mg/kg per day via subcutaneous administration) alone reduced cardiac fibrosis to a greater extent than enalapril (200 mg/L via drinking water; equivalent to 48 mg/kg per day) alone (P<0.05 versus enalapril alone). Additionally, the combined effects of serelaxin and enalapril reduced cardiac fibrosis by at least 2-fold compared with enalapril alone, when administered preventatively or therapeutically; by suppressing transforming growth factor-β1 expression and phosphorylation of Smad2 (an intracellular regulator of transforming growth factor-β1 activity; both P<0.05 versus enalapril alone) to a greater extent. The effects of serelaxin were independent of blood pressure, while enalapril lowered systolic blood pressure in the model studied. These findings suggest that serelaxin alone and in combination with an angiotensin-converting enzyme inhibitor more effectively ameliorates fibrosis than angiotensin-converting enzyme inhibition alone in the diseased heart, in a clinically relevant experimental scenario. (Hypertension. 2014;64:315-322.)

Key Words: angiotensin-converting enzyme inhibitors ■ fibrosis ■ relaxin ■ Smad2 protein ■ transforming growth factor beta1

Fibrosis is defined as the hardening or scarring of tissues attributable to an excessive deposition of extracellular matrix (ECM) components. It results from chronic inflammation and aberrant wound healing and is the final common pathway to almost all forms of progressive cardiovascular disease. Continuous pathological stimuli and various profibrotic cytokines can disrupt the regulatory processes that control the rate of ECM synthesis and degradation. Overabundant ECM production, in particular collagen, the failure to resolve fibrogenesis, and an inability to remove new ECM eventually lead to significant dysfunction and organ failure.

Despite fibrosis being a significant health burden and cause of morbidity and mortality, there are currently no effective treatments that can halt its progression. Indirect strategies aimed at improving the organ environment are nonspecific and have only shown limited success. More direct strategies have attempted to prevent fibrosis by limiting injury, inflammation, and fibrogenesis but are often hampered by the inherent redundancy in cytokine and inflammatory signals. For many years we have known that blocking the renin–angiotensin system leads to a marked reduction in hypertension and, to a lesser extent, direct inhibition of fibrosis progression. However, although targeting angiotensin with converting enzyme inhibition (ACEi) or receptor blockade represents the most advanced therapeutic approach, these therapies only delay end-stage organ failure by months. Hence, novel strategies that target collagen turnover and organization, while augmenting the actions of existing therapeutics (ACEi), may offer significant potential benefits.

The naturally occurring hormone relaxin has emerged as a rapidly occurring antifibrotic that prevents and reduces aberrant collagen deposition and fibrosis progression in a clinically relevant experimental scenario.
various experimental models, regardless of pathogenesis.7–10 Furthermore, it can ameliorate fibrosis in multiple organs undergoing simultaneous aberrant matrix/collagen accumulation.11 The collagen-inhibitory actions of relaxin are mediated by its ability to promote nitric oxide–mediated inhibition of Smad2 phosphorylation (pSmad2; an intracellular protein that promotes transforming growth factor [TGF]-β1 signal transduction)12 and hence the profibrotic actions of TGF-β1.13,14

Recent phase III clinical trials have successfully evaluated the vasodilatory properties of serelaxin (a recombinant form of human gene-2 relaxin, which is the major stored and circulating form of relaxin in humans) in acute heart failure.15 The antifibrotic efficacy of serelaxin, which is bioactive in mice,13,14,16–19 has also been demonstrated experimentally13,14,16–19 and in patients with early symptoms of scleroderma.7–9 Despite the therapeutic potential of serelaxin as an antifibrotic agent, there has been no direct comparison between serelaxin and current standard of care (eg, ACEi). Hence, this study sought to compare the antifibrotic effects of serelaxin to that of enalapril in a murine model of cardiac fibrosis. Additionally, the combined effects of serelaxin and enalapril were assessed to determine whether serelaxin could augment the antifibrotic efficacy of ACEi.

Methods

**Isoproterenol-Induced Cardiac Fibrosis**

Six- to 8-week-old male mice (on a 129sv or 129svxC57B6J background, which are equally sensitive to tissue injury and fibrosis) were used in this study and were housed and maintained under standard conditions. Male mice were used as they generally undergo more severe age- and injury-related cardiac fibrosis compared with their female counterparts.20 The experiments outlined were approved by the animal ethics committees of Monash University and Florey Institute, which adhere to the Australian Code of Practice for the Care and Use of Laboratory Animals for Scientific Purposes.

Subgroups of male mice (n=5–13 per treatment group) were subjected to the isoproterenol model of cardiac injury to assess the antifibrotic efficacy of the individual versus combination therapy. The isoproterenol model was induced as described before,21 with mice given 5 daily subcutaneous injections of isoproterenol hydrochloride (isoproterenol; 25 mg/kg body weight [BW]; Sigma-Aldrich, St Louis, MO), before being left for a further 12 days for left ventricular (LV) fibrosis to develop. Uninjured/untreated mice in addition to isoproterenol-injured/untreated mice served as appropriate controls.

**Treatment Studies**

Dose–response studies for serelaxin (kindly provided by Corthera Inc, San Carlos, CA, a subsidiary of Novartis Pharma AG, Basel, Switzerland) and enalapril (enalapril maleate salt, which is bioactive in mice; Sigma-Aldrich, St Louis, MO)22 were conducted initially to determine a dose for each therapy that maximally inhibited isoproterenol-induced cardiac fibrosis. For these studies, subgroups of mice (n=5–8 per treatment group) were treated with either 0.5, 1, or 2 mg/kg per day of serelaxin via osmotic minipumps (model 2002, with an infusion rate of 0.5 µL per hour; Alzet, Cupertino, CA), or enalapril at 200 (equivalent to 48 mg/kg per day), 300 (72 mg/kg per day), or 500 mg/L (120 mg/kg per day) via drinking water, with all treatments maintained over the 17-day experimental period. The dose range chosen in each case was based on (1) the lowest dose of serelaxin evaluated (0.5 mg/kg per day) having been shown to produce bell-shaped dose–response curves,23 whereby lower physiological responses to the circulating hormone were observed beyond 80 to 100 ng/mL; and (3) the lowest dose of enalapril evaluated (200 mg/L) having been shown to effectively reduce interstitial renal fibrosis.24,25

The optimal doses of serelaxin (0.5 mg/kg per day) and enalapril (200 mg/L) that prevented the isoproterenol-induced cardiac fibrosis were then compared with each other (n=5–7 mice per group) over the 17-day experimental period. Treatment and control arms for both dose–response and comparative studies were run in parallel, with uninjured/untreated and isoproterenol-treated animals being used as relevant controls. Additionally, separate subgroups of isoproterenol-treated mice were coadministered with serelaxin (0.5 mg/kg per day) and enalapril (200 mg/L; n=5–6 mice per group) over the 17 days.

The combined effects of serelaxin (0.5 mg/kg per day) and enalapril (200 mg/L) were also compared with those of enalapril (200 mg/L) alone when treatment was delayed, the more clinically relevant scenario. Isoproterenol-injured mice (n=19) were treated from days 10 to 17 postinjury. Serelaxin was administered via model 1007D osmotic minipumps (Alzet) over the 7-day treatment period, while enalapril was administered via drinking water over the same time period. Uninjured/untreated mice and isoproterenol-injured mice kept for 10 and 17 days (n=6–9 per treatment group and time point) were used as appropriate controls.

**Blood Pressure Measurements**

In all groups, tail cuff plethysmography (MC4000 Blood Pressure Analysis Systems; Hatters Instruments, Inc, NC) was used to measure baseline and end point systolic blood pressures (SBPs) before drug administration (at day 0) and at the completion of the 17-day isoproterenol-induced experimental period (just before animals were culled). At least 15 to 20 measurements per time point were pooled to obtain a mean for each animal.

**Tissue Collection and Analysis**

At the appropriate time points, all uninjured/untreated, isoproterenol-injured/untreated, and drug-treated animals were weighed before being killed by an overdose of anesthesia. The heart and then left ventricle was isolated from all treated and related untreated control animals. LV tissues were transversely divided into 3 portions: the apical region for measuring hydroxyproline content (a measure of collagen content and concentration), the midzone for fixation (10% neutral-buffered formalin), and the basal region for protein extraction and Western blotting of fibrosis-related markers. Unfixed tissue was immediately snap frozen and stored at −80°C. To ensure standardization and enable intergroup comparisons, each assay used the same portion from each animal.

**Hydroxyproline Assay**

Hydroxyproline analysis was carried out on equivalent portions of LV tissues, as described previously.17 Hydroxyproline values were converted to collagen content and then collagen concentration (percent collagen content/dry weight tissue), as described before.17

**Histological Analysis**

Fixed LV sections were paraffin embedded, cut into serial (5 µm) sections, and stained with 0.1% picrosirius red (Polysciences, Inc, Warrington, PA) to identify fibrillar collagen, as detailed previously.19 Images of 6 to 8 nonoverlapping fields from each section were captured and digitalized using an Olympus BX51 microscope with ColourView camera, and Analysis LS Starter software (Olympus, Munster, Germany). All images were collected with an Apochromat 20×0.6 Ph2 objective. Interstitial collagen content (a measure of fibrosis) in the left ventricle was determined by ImageJ software (National Institutes of Health, MD) and expressed as percent collagen staining per total area.

**Immunohistochemistry**

Paraffin-embedded LV serial sections were immunohistochemically stained for TGF-β116 and pSmad218 as described before:
using a polyclonal antibody to TGF-β1 (sc146; 1:200 dilution; Santa Cruz Biotechnology, CA) and monoclonal antibody to pSmad2 (No. 3108; 1:500 dilution; Cell Signaling Technology, MA). Detection of antibody staining was completed using the DAKO EnVision antirabbit kit (DAKO Corp, CA; for TGF-β1) or avidin–biotin complex (ABC Elite; Vector, CA; for pSmad2) and 3,3′-diaminobenzidine (Sigma-Aldrich), with hematoxylin counterstaining of sections. Images of 6 to 8 nonoverlapping fields per section were obtained and quantified as detailed above.

### Western Blotting

Total LV protein from control, isoproterenol-alone, and isoproterenol+treated mice was isolated and equal amounts (containing 15 μg of total protein/lane) were assessed for matrix metalloproteinase (MMP)-13 by Western blotting, as described before, using a primary polyclonal antibody (ab75606; 1:750 dilution; Abcam, Redfern, New South Wales, Australia). Densitometry of the relevant bands was performed using a Bio-Rad GS710 Calibrated Imaging Densitometer and Quantity-One software, and value was expressed relative to value from the uninjured/untreated control group, which was expressed as 1.

### Gelatin Zymography

Gelatin zymography was used to assess changes in LV MMP-2 levels from control, isoproterenol-alone, and isoproterenol+treated mice, as detailed previously. Densitometry of the relevant bands was performed as described above.

### Statistical Analysis

The results obtained were analyzed by 1-way ANOVA with the Newman–Keuls post hoc test used for multiple comparisons between groups (GraphPad Prism 5.03; San Diego, CA). Data are expressed as mean±SEM, with P<0.05 regarded as statistically significant.

### Results

The Relative Antifibrotic Efficacy of Serelaxin and Enalapril in Isoproterenol-Induced Progressive Cardiac Fibrosis

Repeated isoproterenol (25 mg/kg) administration to male 129sv or 129sv×C57B6J mice significantly increased their BWs (by ≈18%), heart weights (HWs, by ≈22%), and LV weights (LVWs, by ≈16%; Table), and induced a marked 2-fold increase in LV collagen concentration (Figure 1A) and an 11- to 12-fold increase in interstitial collagen staining (Figure 1B) by 17 days (all P<0.05 versus respective measurements from uninjured control mice). However, the administration of isoproterenol did not influence SBP (Table).

Serelaxin dose studies demonstrated that none of the evaluated doses (0.5–2 mg/kg per day) affected the isoproterenol-induced increase in BW, HW, LVW (Table), or SBP (Table), with only the 2 mg/kg per day dose of serelaxin affecting LV weight (Table). Enalapril administration (200 mg/L) induced a significant decrease in BW and HW, and the 500 mg/L dose also decreased LV weight (Table). Co-treatment with serelaxin and enalapril resulted in a further decrease in BW, HW, and LVW (Table), with the 2 mg/kg per day dose of serelaxin being the most effective (Table). The combination of serelaxin and enalapril also resulted in a significant decrease in SBP (Table).

### Table. BW, HW, LVW, Organ Weight to BW Ratios, and SBP in Isoproterenol- and Treated-Mice

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>BW, g</th>
<th>HW, mg</th>
<th>LW, mg</th>
<th>HW/BW, mg/g</th>
<th>LW/BW, mg/g</th>
<th>Basal SBP, mm Hg</th>
<th>End SBP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td>22±1</td>
<td>109±2</td>
<td>76±2</td>
<td>5.0±0.1</td>
<td>3.5±0.1</td>
<td>123±3</td>
<td>122±2</td>
</tr>
<tr>
<td>Isoproterenol (5)</td>
<td>26±1*</td>
<td>133±6*</td>
<td>91±1†</td>
<td>4.8±0.1</td>
<td>3.5±0.1</td>
<td>130±3</td>
<td>120±2</td>
</tr>
<tr>
<td>Isoproterenol+0.5 mg/kg per day serelaxin (5)</td>
<td>25±1*</td>
<td>133±4*</td>
<td>87±3*</td>
<td>5.3±0.1</td>
<td>3.5±0.1</td>
<td>127±6</td>
<td>130±3</td>
</tr>
<tr>
<td>Isoproterenol+1 mg/kg per day serelaxin (6)</td>
<td>25±1*</td>
<td>135±4*</td>
<td>94±4†</td>
<td>5.4±0.1</td>
<td>3.6±0.1</td>
<td>123±6</td>
<td>130±5</td>
</tr>
<tr>
<td>Isoproterenol+2 mg/kg per day serelaxin (5)</td>
<td>25±1*</td>
<td>135±4*</td>
<td>91±4†</td>
<td>5.3±0.1</td>
<td>3.6±0.1</td>
<td>123±6</td>
<td>130±5</td>
</tr>
<tr>
<td>Isoproterenol+200 mg/L enalapril (5)</td>
<td>24±1</td>
<td>123±6</td>
<td>81±2†</td>
<td>5.1±0.1</td>
<td>3.4±0.2</td>
<td>130±4</td>
<td>107±4§</td>
</tr>
<tr>
<td>Isoproterenol+300 mg/L enalapril (6)</td>
<td>22±1†</td>
<td>100±2†</td>
<td>65±2†</td>
<td>4.5±0.1†</td>
<td>3.0±0.1†</td>
<td>129±6</td>
<td>95±7†**</td>
</tr>
<tr>
<td>Isoproterenol+500 mg/L enalapril (6)</td>
<td>21±1†</td>
<td>92±3†**</td>
<td>56±3†**</td>
<td>4.4±0.1†</td>
<td>2.7±0.1†</td>
<td>124±6</td>
<td>94±7†**</td>
</tr>
<tr>
<td>Isoproterenol+0.5 mg/kg per day serelaxin+200 mg/L enalapril (5)</td>
<td>27±1*</td>
<td>122±6</td>
<td>84±3</td>
<td>4.5±0.2</td>
<td>3.1±0.1</td>
<td>132±3</td>
<td>112±6§</td>
</tr>
<tr>
<td>Isoproterenol+200 mg/L enalapril [D] (6)</td>
<td>24±1</td>
<td>122±5</td>
<td>80±2†</td>
<td>5.1±0.2</td>
<td>3.4±0.1</td>
<td>128±5</td>
<td>107±3§</td>
</tr>
<tr>
<td>Isoproterenol+0.5 mg/kg per day serelaxin+200 mg/L enalapril [D] (6)</td>
<td>24±1</td>
<td>125±6</td>
<td>87±3*</td>
<td>5.2±0.2</td>
<td>3.6±0.1</td>
<td>129±2</td>
<td>107±2§</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SEM; numbers in parenthesis represent the number of animals analysed per treatment group. [D] indicates delayed treatment (from day 10 to 17); BW, body weight; HW, heart weight; LW, left ventricular weight; and SBP, systolic blood pressure.

*P<0.05 vs control group.
†P<0.01 vs control group.
‡P<0.05 vs isoproterenol group.
§P<0.05 vs respective basal SBP measurements.
||P<0.01 vs isoproterenol group.
¶P<0.01 vs isoproterenol+200 mg/L enalapril group.
**P<0.001 vs isoproterenol group.
††P<0.05 vs isoproterenol+200 mg/L enalapril group.
but consistently inhibited the isoproterenol-induced increase in LV collagen concentration (Figure 1A) and interstitial collagen staining (Figure 1B) by 50% to 60% (all $P<0.01$ versus isoproterenol alone–treated group). Hence, the lowest dose of 0.5 mg/kg per day was used for subsequent comparative purposes.

In comparison, the enalapril dose–response studies demonstrated that the lowest dose evaluated (200 mg/L) was able to normalize the isoproterenol-induced effects on BW, HW, and LVW back to that measured in uninjured mice (Table), while significantly inhibiting the isoproterenol-induced increase in collagen concentration (Figure 1C) and staining (Figure 1D) by 25% to 35% (both $P<0.05$ versus isoproterenol alone–treated group), and significantly reducing final SBP in isoproterenol–treated mice by $\approx 15\%$ ($P<0.05$ versus basal SBP; Table). Higher doses of the ACEi (300 and 500 mg/L) further reduced SBP (by $\approx 25\%$; $P<0.05$ versus basal SBP from respective groups and end SBP measured from isoproterenol–injured mice; Table), abrogated the isoproterenol–induced increase in BW (Table), and dose dependently reduced HW and LVW to values that were below that obtained from uninjured control mice (all $P<0.05$ versus control mice; $P<0.01$ versus isoproterenol–injured and isoproterenol+200 mg/L–treated mice; Table). Although these higher doses of enalapril reduced absolute LV collagen levels, when expressed as a percentage of the smaller dry weight tissues, they did not further reduce LV collagen concentration (Figure 1C) or staining (Figure 1D). Hence, a dose of 200 mg/L was chosen for subsequent studies.

When the effects of serelaxin (0.5 mg/kg per day) were directly compared with those of enalapril (200 mg/L) in preventing the effects of isoproterenol–induced injury, serelaxin was found to significantly prevent the isoproterenol–induced increase in LV collagen concentration (by $\approx 55\%$; $P<0.01$ versus isoproterenol alone; Figure 2A) to a greater extent than enalapril alone (by $\approx 25\%$; $P<0.05$ versus isoproterenol alone; $P<0.05$ versus isoproterenol+enalapril). Likewise, serelaxin was found to significantly prevent interstitial collagen staining (by $\approx 61\%$; $P<0.01$ versus isoproterenol alone; Figure 2B) to a greater extent than enalapril (by $\approx 40\%$; $P<0.05$ versus isoproterenol alone). The collagen–inhibitory effects of serelaxin were independent of any changes on animal BW, HW, LVW, or SBP.

The Effects of Combination Treatment in Preventing Isoproterenol–Induced Cardiac Fibrosis Progression

Combination therapy with serelaxin (0.5 mg/kg per day) and enalapril (200 mg/L) did not significantly affect the isoproterenol–induced increase in BW (Table) but normalized HW and LVW back to levels measured in control mice (Table). This combination therapy also significantly reduced SBP (by 13%–15%; $P<0.05$ versus basal SBP; Table) and prevented isoproterenol–induced LV collagen concentration (Figure 2A) by $\approx 68\%$, which was double that of the effects of enalapril alone ($P<0.001$ versus isoproterenol alone; $P<0.01$ versus isoproterenol+enalapril) but not different from the effects of serelaxin alone. Combination of
serelaxin and enalapril lowered isoproterenol-induced interstitial LV collagen staining (by ≈67%; Figure 2B) to a significantly greater extent than enalapril alone (P<0.05 versus isoproterenol+enalapril), but not to the extent when serelaxin was used alone.

The Effects of Serelaxin, Enalapril, and Combination Therapy on Fibrogenic and Fibrolytic Pathways

The ability of serelaxin alone to prevent isoproterenol-induced fibrosis progression was associated with its enhanced ability to downregulate TGF-β1 expression (by ≈80%; Figure 3A) and pSmad2 (by ≈77%; Figure 3B; both statistically not different from control group values) over the effects of enalapril alone (which only prevented aberrant TGF-β1 and pSmad2 levels by ≈43% and ≈33%, respectively; both P<0.05 versus isoproterenol+enalapril).

The combination of serelaxin and enalapril also suppressed TGF-β1 expression (by ≈80%; Figure 3A) and pSmad2 (by ≈85%; Figure 3B) to an extent similar to that suppressed by serelaxin alone, but by at least 2-fold when enalapril was used alone (both P<0.05 versus isoproterenol+enalapril).

Serelaxin also stimulated MMP-13 (by ≈1-fold; P<0.01 versus control group) and MMP-2 (by ≈80%; P<0.05 versus control group) levels above that measured in uninjured/control animals (Figure 3C), but this was no different from that induced by isoproterenol+enalapril treatment (for MMP-13 and MMP-2) or isoproterenol treatment alone (for MMP-2). Combination therapy also stimulated MMP-13 and MMP-2 levels to an extent similar to that stimulated by serelaxin or enalapril alone (Figure 3C).

The Effects of Delayed Serelaxin and Enalapril Combination Therapy in Reducing Established Cardiac Fibrosis

Given that serelaxin in combination with enalapril was able to prevent isoproterenol-induced cardiac fibrosis (Figure 2) 2-fold compared with enalapril alone when treatment was initiated at the onset of injury, the delayed effects of this combination therapy were compared with those of enalapril alone in the isoproterenol model (Figure 4). Isoproterenol induced a ≈1.1-fold and ≈2-fold increase in LV collagen concentration (fibrosis) by days 10 and 17 postinjury, respectively (both P<0.001 versus control mice; Figure 4). When administered from days 10 to 17 postinjury, mean LV collagen concentration levels in enalapril alone–treated mice were 17% to 20% lower but not significantly different from that measured from day 17 post-isoproterenol–injured animals. In contrast, the combined effects of serelaxin and enalapril significantly ameliorated the difference in LV collagen concentration, between days 10 and 17, by ≈72% (P<0.01 versus isoproterenol alone on day 17; P<0.05 versus isoproterenol+enalapril on day 17; no difference to isoproterenol alone on day 10; Figure 4), in the presence of a ≈15% reduction in SBP.

Discussion

The widespread recognition that fibrosis is the final common pathway to progressive organ failure, and the limited efficacy of available therapies, has inevitably led to a search for new rationale treatment strategies. In recent studies, we and others have consistently shown that serelaxin is a potent antifibrotic in a wide variety of experimental models and scenarios. Although these studies are important proof of principle, preclinical validation requires a number of additional steps including head-to-head comparison with standard care and validation of therapeutic rather than preventative strategies. These issues are specifically addressed in this study. Our main findings were that the antifibrotic efficacy of serelaxin was greater than that of enalapril in isolation in the diseased heart, suggesting that serelaxin may be a better alternative to ACEi as an effective treatment for nonhypertensive heart disease. Furthermore, serelaxin in combination with ACE inhibition effectively reduced fibrosis progression to a greater extent than ACE inhibition alone, when administered after fibrosis was established, indicating that this combination therapy would be more effective in treating hypertension-related fibrosis, while retaining the blood pressure–lowering effects of the ACEi.
Our initial dose finding studies were based on previously published antifibrotic efficacy of these agents\textsuperscript{11,13,16–19,22,25} and were designed to find a point at which the antifibrotic effects of each compound plateaued. These preliminary studies showed that 0.5 mg/kg per day of serelaxin and 200 mg/L of enalapril inhibited cardiac fibrosis, and in each case there was no additional effect at higher doses. Importantly, when administered at a dose of 0.5 mg/kg per day, serelaxin had also previously been shown to reduce LV end diastolic pressure and myocardial stiffness, while increasing ventricular contractility in various experimental rodent models of heart disease\textsuperscript{27,28}, suggesting that the circulating levels it produced\textsuperscript{23} could inhibit fibrosis progression and improve related cardiac function.

To our knowledge, this is the first study to directly compare the antifibrotic efficacy of serelaxin to an ACEi in an experimental setting. Serelaxin was found to prevent isoproterenol-induced LV fibrosis, and when administered at a dose of 0.5 mg/kg per day, prevented the isoproterenol-induced cardiac fibrosis to a significantly greater extent than enalapril (200 mg/L). Furthermore, the combined effects of serelaxin and enalapril prevented isoproterenol-induced cardiac fibrosis by at least 2-fold compared with enalapril alone, when treatment was initiated before injury. Given that the dose–response studies suggested a plateau in the antifibrotic efficacy of ACE blockade from 200 mg/L, the additional antifibrotic effects seen with serelaxin are likely mediated at a downstream level of ACE blockade to abrogate the profibrotic influence of TGF-β1.
The magnitude of changes seen in collagen with picrosirius red staining was greater than that seen by measuring hydroxyproline content, which is consistent with similar studies in other models. A number of factors may be relevant, which include, but are not limited to, scale (extrapolation of a single tissue section area to volume); correction for dry weight (biochemical analysis accounts for hypertrophy and atrophy, whereas histology does not); the extent to which fine collagen fibers can be detected; and a propensity for picrosirius red to overestimate collagen. Importantly though, the same trends in injury- and drug-induced changes in collagen were consistently observed from the biochemical and histological assays performed.

TGF-β signal transduction promotes fibrosis progression at multiple levels, by enhancing myofibroblast differentiation and collagen synthesis and deposition, while reducing ECM/collagen degradation through inhibition of MMPs. The antifibrotic actions of ACEi are attributed to both indirect hemodynamic effects and direct inhibition of TGF-β1 activity. However, consistent with previous findings in the kidney, even at maximally effective doses, enalapril failed to normalize TGF-β1 production and matrix accumulation in the injured heart. Likewise, antifibrotic properties of serelaxin have consistently been shown to be mediated by inhibiting aberrant TGF-β1 signaling in progressive fibrosis. The improved antifibrotic efficacy of serelaxin over enalapril in the setting of cardiac injury was primarily associated with its increased ability to suppress TGF-β1 expression and signal transduction (via inhibition of pSmad2), and hence the downstream effects of TGF-β1 activity on collagen kinetics. Furthermore, the findings obtained from the isoproterenol model add to recent literature in demonstrating that serelaxin primarily disrupts TGF-β1 signal transduction in the heart by inhibiting the phosphorylation and translocation of Smad2.

Pharmacological fibrolysis, through MMP activation, is a counterbalance to fibrogenesis, and may in some circumstances limit matrix accumulation. It is, however, also a feature of injury itself, where activation of MMPs is thought to be a physiological attempt to limit excess matrix. The isoproterenol-induced promotion of MMP-13 and MMP-2 is consistent with this, with both serelaxin and enalapril producing a modest increase in MMP-13 over the effects of injury alone, in the absence of any added effects on cardiac MMP-2 levels. These results suggested that the improved antifibrotic efficacy of serelaxin and combination therapy over that of enalapril alone was not explained by changes in MMP-induced collagen degradation and that the drug-induced increases in MMP levels measured did not appear to play a significant role in limiting fibrosis progression in the model studied.

A deficiency in many studies of putative antifibrotics is that they only examine preventative strategies, that is, when administration is started before or at the onset of injury. In the current study, we examined the combined effects of serelaxin and enalapril, in comparison with enalapril alone, on progressive cardiac fibrosis when treatment was delayed until fibrosis was established. In the model studied, combination therapy abrogated further progression, which enalapril alone did not achieve. As ACE inhibitors alone have only modestly prevented organ failure and mortality when administered after injury onset, serelaxin may act as a suitable adjunct therapy to ACE inhibition to further abrogate established scarring without affecting the antihypertensive effects of ACEi.

In conclusion, the findings of the current study demonstrated that serelaxin alone or in combination with enalapril had improved antifibrotic efficacy compared with ACEi alone. Thus, serelaxin is a more effective antifibrotic and suppressor of TGF-β1 activity compared with enalapril in the setting of heart failure. Additionally, serelaxin and ACEi in combination effectively reduced established fibrosis when treatment was delayed, suggesting that serelaxin may also augment the antifibrotic actions of front-line therapies that target renin-angiotensin system and TGF-β1 activity.

**Perspectives**

Although angiotensin blockade has recognized direct antifibrotic effects, ACEi only delay the progression of organ fibrosis by months and can have undesirable side effects when chronically administered. The increased antifibrotic efficacy of serelaxin seen here in the injured heart makes it a highly desirable therapeutic agent for the attenuation of organ fibrosis. Our novel findings demonstrated that serelaxin targeted the TGF-β1/Smad2 axis to a greater extent than enalapril and also effectively ameliorated organ fibrosis when treated in combination with enalapril. These findings suggest that serelaxin is a potent antifibrotic agent in isolation and can augment the antifibrotic utility of ACEi in hypertensive disease.

**Sources of Funding**

This work was supported by Monash Mid-Career and National Health and Medical Research Council (NHMRC) of Australia.
Senior Research Fellowships to C.S. Samuel; and by the Victorian Government’s Operational Infrastructure Support Program.

Disclosures
None.

References

Novelty and Significance

What Is New?
• The experimental antifibrotic efficacy of serelaxin was compared with a clinically used angiotensin converting enzyme inhibitor for the first time.

What Is Relevant?
• Serelaxin has potent and rapid antifibrotic actions in the setting of non-hypertensive cardiovascular disease.
• Serelaxin can also effectively attenuate existing fibrosis in combination with angiotensin converting enzyme inhibitor, which should have important implications for the treatment of hypertension.

Summary
Serelaxin alone or in combination with enalapril had improved antifibrotic efficacy compared with the angiotensin converting enzyme inhibitor alone, even when treatment was delayed. The improved antifibrotic efficacy of serelaxin over enalapril was associated with its greater ability to suppress transforming growth factor-β1 expression and signal transduction. Serelaxin may augment the antifibrotic actions of front-line therapies that target the renin–angiotensin system and transforming growth factor-β1 activity.
Serelaxin Is a More Efficacious Antifibrotic Than Enalapril in an Experimental Model of Heart Disease
Chrishan S. Samuel, Hasangika Bodaragama, Jacqueline Y. Chew, Robert E. Widdop, Simon G. Royce and Tim D. Hewitson

Hypertension. 2014;64:315-322; originally published online May 27, 2014;
doi: 10.1161/HYPERTENSIONAHA.114.03594

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/64/2/315

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/