Relaxin Suppresses Atrial Fibrillation by Reversing Fibrosis and Myocyte Hypertrophy and Increasing Conduction Velocity and Sodium Current in Spontaneously Hypertensive Rat Hearts


Rationale: Atrial fibrillation (AF) contributes significantly to morbidity and mortality in elderly and hypertensive patients and has been correlated to enhanced atrial fibrosis. Despite a lack of direct evidence that fibrosis causes AF, reversal of fibrosis is considered a plausible therapy.

Objective: To evaluate the efficacy of the antifibrotic hormone relaxin (RLX) in suppressing AF in spontaneously hypertensive rats (SHR).

Methods and Results: Normotensive Wistar-Kyoto (WKY) and SHR were treated for 2 weeks with vehicle (WKY+V and SHR+V) or RLX (0.4 mg/kg per day, SHR+RLX) using implantable mini-pumps. Hearts were perfused, mapped optically to analyze action potential durations, intracellular Ca²⁺ transients, and restitution kinetics, and tested for AF vulnerability. SHR hearts had slower conduction velocity (CV; \( P<0.01 \) versus WKY), steeper CV restitution kinetics, greater collagen deposition, higher levels of transcripts for transforming growth factor-β, metalloproteinase-2, metalloproteinase-9, collagen I/III, and reduced connexin 43 phosphorylation (\( P<0.05 \) versus WKY). Programmed stimulation triggered sustained AF in SHR (n=5/5) and SHR+V (n=4/4), but not in WKY (n=0/5) and SHR+RLX (n=1/8; \( P<0.01 \)). RLX treatment reversed the transcripts for fibrosis, flattened CV restitution kinetics, reduced action potential duration at 90% recovery to baseline, increased CV (\( P<0.01 \)), and reversed atrial hypertrophy (\( P<0.05 \)). Independent of antifibrotic actions, RLX (0.1 pmol/L) increased Na⁺ current density, \( I_{Na} =2 \text{-fold in } 48 \text{ hours} \) in human cardiomyocytes derived from inducible pluripotent stem cells (n=18/18; \( P<0.01 \)).

Conclusions: RLX treatment suppressed AF in SHR hearts by increasing CV from a combination of reversal of fibrosis and hypertrophy and by increasing \( I_{Na} \). The study provides compelling evidence that RLX may provide a novel therapy to manage AF in humans by reversing fibrosis and hypertrophy and by modulating cardiac ionic currents. (Circ Res. 2013;113:313-321.)

Key Words: atrial fibrillation ■ fibrosis ■ hypertrophy ■ \( I_{Na} \) upregulation ■ optical mapping, action potential ■ relaxin ■ spontaneously hypertensive rats

Atrial fibrillation (AF), a disease associated with mortality, morbidity, and high costs, affects tens of millions of people worldwide and is increasing in prevalence. Among the many risk factors that promote the development of AF, the most prominent are sex (more prevalent in men than women), old age (>60 years old), and hypertension. Hypertension and aging lead to structural changes of the extracellular matrix (ECM) and enhanced AF vulnerability as a result of the altered myocardial substrate. Another cause of AF is atrial tachycardia that leads to electric remodeling and altered intracellular Ca²⁺ homeostasis associated with decreases in action potential duration (APD) and shortened atrial refractory periods (RPs). Fibrosis is a hallmark of arrhythmogenic ECM remodeling, occurs with alterations in connexin expression, and slows conduction velocity (CV), creating a barrier to impulse propagation by disrupting intermyocyte coupling.
Increased collagen deposition has been well documented in AF patients compared with control subjects. Although the precise signaling processes of fibrosis are unknown, multiple factors have been implicated (e.g., angiotensin II, transforming growth factor-β [TGF-β1], and platelet-derived growth factor) in the pathogenesis of atrial fibrosis. Angiotensin-converting enzyme overexpression is associated with atrial enlargement, atrial fibrosis, and AF, whereas blockade of angiotensin-converting enzyme blunts atrial fibrosis and AF in animal models and patients with heart failure. TGF-β1 and platelet-derived growth factor are thought to act on cardiac fibroblasts to increase collagen production without offsetting increases in collagen degradation. It should be noted that the role of fibrosis as the cause of AF can be overstated because some studies show no difference in fibrosis in AF and control patients. A possible explanation that remains unproven is that only some forms of collagen deposition cause AF, namely interstitial and disorganized collagen deposition promotes AF rather than surface collagen.

Current modalities for suppression of AF include drugs and ablation, each of which is limited by inefficacy, intolerance, and toxicity. Current drugs do not fundamentally alter the atrial substrate, whereas ablation requires destruction of viable tissue. Complications, costs, and difficulties associated with ablation have encouraged the development of safer drug therapies for the treatment of AF. Existing antiarrhythmic drug approaches have limited effectiveness and are associated with risks of serious complications, particularly ventricular proarrhythmia and organ toxicity. The spontaneously hypertensive rat (SHR) has been widely studied as model of the effects of hypertension on the cardiovascular system. In SHR, hypertension progresses as a function of age, is more pronounced in males than females, and exhibits most of the hallmarks of the human disease. Previous studies on the SHR model have shown an increased incidence of AF and atrial tachyarrhythmias compared with normotensive Wistar-Kyoto (WKY) rats, attributed to greater levels of fibrosis. These findings suggest that fibrosis may promote the development of AF, making it an important antiarrhythmic target.

Relaxin (RLX), a pleiotropic hormone, which is widely conserved, has been shown to have a wide range of biological actions, including anti-inflammatory, antiapoptotic, cardioprotective, vasodilatory, proangiogenic effects, and antifibrotic effects. RLX was first identified for its role in reproduction and pregnancy. It is thought to play a critical role in the hemodynamic adaptive and antifibrotic changes that occur during pregnancy. Male RLX gene-deficient mice developed age-related cardiac fibrosis, ventricular stiffening, and diastolic dysfunction, suggesting an important role as an intrinsic regulator of collagen turnover.

In the present report, we demonstrate that exogenous systemic administration of RLX to SHRs suppresses AF inducibility by reversing fibrosis and hypertrophy and by increasing CV. These actions of RLX may be relevant to human AF, and as a proof of concept, we show that RLX upregulates $I_{\text{Na}}$ in human cardiomyocytes derived from inducible pluripotent stem cells (iPS-CMs) by a genomic mechanism.

### Methods

#### Study Design

All animals received humane care in a facility, in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication 85-23, revised 1985). The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. AF inducibility was studied in age-matched (9–12 months) and sex-matched (male) rats (Charles River Laboratories) that were separated into 4 groups: (1) normotensive WKY untreated rats; (2) untreated SHR; (3) SHR treated with the vehicle saline (SHR+V); and (4) SHR treated with RLX (SHR+RLX). Recombinant human RLX was supplied by Corthera-Novartis (Basel, CH). Osmotic mini-pumps (ALZET; Durect Corporation, model 2ML2) were used for the RLX and V treatment groups. Pumps were loaded with either recombinant human RLX solution (1.67 mg/mL) or V (20 mmol/L sodium acetate buffer, pH 5.0). The RLX infusion rate was 0.5 mg/kg per day (for 400 g rats) during the 14-day period. This dose of RLX is comparable with the dose previously used to treat in vivo rodent models of fibrosis and to examine effects of RLX on arterial hemodynamics and vascular mechanical properties. Pumps were surgically implanted using sterile technique into the subcutaneous space on the left side of anesthetized animals. Animals were monitored during the 14 days of RLX or V delivery to confirm proper healing of the implant pocket. Experiments showed that rats treated with the saline vehicle had, as expected, similar electrophysiological properties as untreated rats, and as stated, data from the 2 groups were combined into some figures, which also allowed us to display the findings more clearly. For Western blot and reverse transcriptase polymerase chain reaction (RT-PCR) analysis, the 4 groups were WKY treated with vehicle (WKY+V) or relaxin (WKY+RLX) and SHR treated with vehicle (SHR+V) or relaxin (SHR+RLX).

#### Physiological Measurements

Blood pressure, heart rate, and serum RLX concentration were measured at 3 time points of the treatment as described in the Online Data Supplement: pretreatment (day 0), mid-treatment (day 7), and posttreatment (day 14). Hearts were perfused in a Langendorff apparatus to map action potentials (APs) and intracellular Ca$^{2+}$ transients simultaneously using standard techniques (see Online Data Supplement).

Programmed stimulation was used to test AF vulnerability; each heart was paced at the right atrium (RA) using a stimulation protocol consisting of 20 S1 pulses at 250 ms cycle length, followed by a premature S2 pulse (see Online Data Supplement). Maps of APs were used to calculate CV, generate activation maps, measure APD$_{90}$, and investigate the nature of AF by time and frequency domain analysis using previously reported techniques (see Online Data Supplement). Transient AF lasted <3 seconds and self-terminated, whereas sustained AF lasted >3 minutes and was terminated by a bolus injection of KCl (1 mol/L) in the compliance chamber located above the aortic cannula to the heart.
Tissue Analysis
Atrial tissues were used to investigate changes in collagen deposition, connexin 43 phosphorylation, hypertrophy of cardiomyocytes, and transcripts for fibrosis as described in the Online Data Supplement. RT-PCR analysis was used to measure the expression levels of RNAs of interest, which were normalized to GAPDH. Primer pair sequences (forward and reverse for each target, listed 5' to 3') used for RT-PCR are given in the Online Data Supplement for matrix metalloproteinase (MMP)-2, collagen I, collagen III, TGF-β, and GAPDH.

Statistics
AF vulnerability between the different groups was compared using Fisher exact test. Parameters recorded under different S1–S2 were compared using ANCOVA. For RT-PCR, Western blot, and immunofluorescence microscopy, comparisons among ≥3 groups were performed using a nonparametric test (Kruskal–Wallis) with post hoc analyses (Conover). All results are reported as means±SD unless otherwise stated. For all tests, a value of P<0.05 was considered to be statistically significant.

Results
AF Vulnerability
AF was inducible in each of the 5 SHR animals, but none of the 5 WKY animals (P<0.01; Figure 1). In WKY hearts, a premature impulse close to the RP (S1–S2=50 ms) captured and propagated, whereas still shorter intervals (S1–S2 <50 ms) failed to capture and did not induce AF (n=0/5; Figure 1Aa and 1Ab).

In SHR hearts (Figure 1B), a premature impulse at S1–S2 of 75 ms captured and propagated normally (a), but a 70-ms S1–S2 interval induced a transient arrhythmia (b), and a still shorter interval produced sustained AF (c and d; n=5/5; P<0.01 versus WKY). In left atria (LA) while pacing at 250 ms cycle length, RPs were shorter than mean APD90 (WKY: RP=40±13 ms, APD90=98±18 ms, n=5, P<0.05; SHR: RP=58±10 ms, mean APD90=87±18 ms, n=5, P<0.05). RPs were shorter in WKY versus SHR atria (n=5 each; P=0.01), and in SHR hearts, sustained AF was initiated at S1–S2 of 70±12 ms, which was not significantly different from their mean RP (n=5; P=NS).

Optical Mapping of AF
Figure 2 illustrates AP from an SHR heart before and during a transient AF (A) and during a sustained AF (B). Activation maps during transient (a–g) AF (A) exhibited a stable reentry pattern, with wavefronts emanating from a similar origin and propagating in a similar direction from beat to beat. In contrast, during sustained AF (Figure 2B′–2B′g), the origins of successive reentrant waves varied randomly and the arrhythmia was perpetuated by coexisting reentrant circuits maintained through the continuous annihilation and creation of daughter wavelets.27 Voltage oscillations during AF were analyzed in time and frequency domains to visualize the evolution of AF frequencies.27 The spectrogram (short-time Fourier transform) reveals coexisting reentrant circuits at different frequencies (9–20 Hz) and energy densities (Figure 2C). The analysis showed that the RA and LA had similar dominant frequencies (13.7±1.4 and 14.2±0.8 Hz; Figure 2D). In SHR hearts, abnormalities in Ca2+ homeostasis (eg, larger intracellular free Ca2+ transients [Ca2+]i and spark amplitudes, normal L-type Ca2+ current density, Ica,L, and absence of heart failure) have been attributed to cellular hypertrophy, resulting in altered coupling between Ca2+ entry via Ica,L and sarcoplasmic reticulum Ca2+ release.28 The altered sarcoplasmic reticulum Ca2+ release in SHR hearts suggested a potential mechanism to initiate and sustain AF, which we tested by simultaneous mapping of APs and Ca2+Ts to search for spontaneous (nonvoltage-dependent) Ca2+ release and Ca2+ oscillations. As shown in the Online Data Supplement (Online Figure IIA), Ca2+ followed membrane potential (Vn) during transient arrhythmia and sustained AF (Online Figure IIB); neither did Ca2+ oscillations occur that were not associated with voltage depolarizations (n=4/4 hearts).

Effects of RLX Treatment on Blood Pressure, Heart Rate, Serum RLX, and AP
RLX was not detectable in the serum of animals, unless administered exogenously. In SHR+RLX rats, serum RLX measured on the final day of treatment was 70±9 ng/mL, whereas SHR+V rats had undetectable levels of RLX (P<0.001, see Online Figure IS). Blood pressures were comparable between SHR+RLX and SHR+V animals at all time points, indicating that RLX did not reverse the hypertension (Online Table I).

RLX is known to cause an acute increase in heart rate, mediated by cAMP elevation consistent with our findings that RLX (100 nmol/L) perfusion increased heart rate by 10% to 15% within a minute (n=5 per group: SHR or WKY). A similar
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increase in heart rate was found in SHRs in mid-treatment (1 week) and post-treatment (2 weeks) with RLX (Table I). The effects of RLX or V treatment on APD 90, CV, and AP rise time on the LA of SHR hearts were measured as a function of cycle length and compared with values measured in untreated SHR and WKY hearts. These electric characteristics are shown for the LA in the Table, whereas the heart was paced on the RA. APD 90 were shorter in SHR than WKY (P < 0.05), shorter in SHR+V than SHR (P < 0.05), and shorter in SHR+LX than SHR+V (P < 0.01) using ANCOVA.

CV was slower in SHR and SHR+V than WKY hearts, and SHR+RLX resulted in a marked increase in CV compared with WKY, SHR, and SHR+V (P < 0.005; Table). AP rise times tended to be shorter in SHR than WKY hearts, and SHR+RLX tended to reduce rise times further, but these changes did not reach statistical significance. While pacing on the RA, similar results were obtained on right atria (Online Table II). The shape and time course of APs from WKY, WKY+RLX, SHR, and SHR+RLX hearts are illustrated in Online Figure IB.

Table. Effect of RLX on APD 90, CV, and AP Rise Time vs CL in Left Atria

<table>
<thead>
<tr>
<th>CL</th>
<th>WKY (n=5)†</th>
<th>SHR (n=5)</th>
<th>SHR+V (n=5)*</th>
<th>SHR+RLX (n=5)*,‡</th>
<th>CV</th>
<th>SHR+V</th>
<th>SHR+RLX‡</th>
<th>Rise Time</th>
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<td>85±12</td>
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In each left atria, AP rise time and APD 90 were measured from 10 pixels and averaged for 5 atria; CL, AP rise time, and APD 90 were in ms, CV in m/s, as means±SD. For WKY, SHR, and SHR+RLX, n=5 hearts and for SHR+V, n=4 hearts. AP indicates action potential; APD, AP durations; CL, cycle length; CV, conduction velocity; RLX, relaxin; SHR, spontaneously hypertensive rat; V, vehicle; and WKY, Wistar-Kyoto.

*P<0.05 vs SHR.
†P<0.05 vs WKY, SHR, and SHR+V (ANCOVA).
Effect of RLX on AF Inducibility
A major and consistent finding was that RLX treatment of SHR for 2 weeks suppressed AF inducibility (n=7/8; 1 heart had an infarct; Figure 3A and 3B). In contrast, V treatment of SHR failed to suppress AF inducibility (n=4/4; P<0.01 versus SHR+RLX; Figure 3C and 3D). More robust attempts to elicit AF in RLX-treated SHR hearts, such as varying the location of the pacing electrode and burst pacing (10 stimuli, 10-ms apart) on either the RA or LA, failed to elicit AF. In rare cases, the S2 impulse produced a nonsustained arrhythmia of <10 beats (Figure 3A).

The mean RFs for SHR+V (51±4.3 ms; n=4) and SHR+RLX (50±10 ms; n=5) LA were not significantly different (P=NS). CV and APD restitution kinetics (RK) were measured from the RA and LA of WKY, SHR (untreated and treated with vehicle were combined), and SHR+RLX. Figure 4 (right) shows a marked effect of RLX on the CV RK of LA and RA compared with SHR hearts—namely, a large increase in CV particularly for short S1–S2 intervals and a less- steep RK curve. RLX treatment did not significantly alter the slope of APD RK curves (left) for LA and RA. RLX-treated SHR hearts had shorter APD90 RK curves compared with SHR+V and WKY hearts, consistent with APD90 in the Table. Activation maps of paced beats (S1), the premature beat (S2), and the first spontaneous beat are shown for an SHR+V and an SHR+RLX atrium (Figure 3A and 3B). The slower CV of the premature impulse and of the first spontaneous reentrant beat in SHR+V atria helps to sustain AF.

Histologic Findings
Differences in the level of fibrosis in the LA and RA of the different groups are shown in Figure 5. SHR had a significantly greater collagen-to-tissue ratio in both the RA and LA compared with WKY (P<0.05). There was no significant difference in collagen-to-tissue ratio in both the RA and LA between SHR and SHR+V. However, RLX treatment attenuated the fibrosis within 2 weeks because SHR+RLX had a significantly lower collagen-to-tissue ratio compared with SHR and SHR+V (P<0.05). SHR+V LA cardiomyocytes had a significant level of hypertrophy, with greater cross-sectional area of LA myocytes (cross-sectional area=146.9±07.2 µm²) compared with WKY+V (95.±10.6 µm²; P<0.01). The cross-sectional area of WKY+RLX atrial myocytes (96.9±3.3 µm²) did not differ from that of WKY+V. However, the cross-sectional area of LA cardiomyocytes from SHR+RLX was significantly less (100.8±2.98 µm²; P<0.05) than that of SHR+V and not significantly different from either WKY group. Thus, RLX seemed to reverse atrial myocyte hypertrophy in SHR hearts.

Effect of RLX on Connexin 43 Phosphorylation and Fibrosis-Related Transcripts
The effect of RLX treatment on the relative phosphorylation of connexin 43 in SHR RA was assessed by Western blot analysis, using the differential molecular weight of phosphorylated (43 kDa) to nonphosphorylated connexin 43 (40 kDa). Proteins from relaxin-treated SHR showed a significantly greater ratio in the band intensity of the 43- to 40-kDa proteins (SHR+RLX, 5.74±1.46; SHR+V, 2.15±1.26; n=4/group; P<0.01). The effect of RLX versus V treatment on fibrosis-related transcripts was examined by RT-PCR from RNA isolated from the LA of 4 to 5 rats per group (WKY+V, WKY+RLX, SHR+V, SHR+RLX; Figure 6). TGF-β, MMP-2, MMP-9, collagen I, and collagen III transcripts were all significantly elevated in SHR+V versus WKY+V (P≤0.05). In WKY, RLX treatment did not alter fibrosis-related transcripts (Figure 6). In contrast, SHR+RLX treatment significantly reduced all the transcripts, except for collagen III, which exhibited a marked trend toward a decrease. For TGF-β, MMP-2, and MMP-9, transcripts levels in SHR+RLX were not different from those in WKY+V or WKY+RLX groups. Collagen I transcripts levels, while significantly reduced relative to SHR+V, remained somewhat elevated relative to WKY groups. Collagen III transcripts followed a similar pattern.

RLX Upregulates I Na in Human iPS-CMs Independent of Fibrosis
A main electrophysiological change caused by RLX is a marked increase in CV, which is difficult to attribute solely to reduced fibrosis and altered expression, localization, and phosphorylation of connexin 43. Alternatively, large increases in CV are more readily caused by an increase in current density of voltage-gated sodium channels, I Na. To test the effects of RLX on I Na and the relevance of our findings in rat hearts to human hearts, we tested the effects of RLX on I Na density in human iPS-CMs. Human iPS-CMs were cultured with vehicle or 0.1 µmol/L RLX for 48 hours, and then I Na density was measured using the whole-cell voltage-clamp technique (see Methods in the Online Data Supplement). Treatment of human iPS-CMs with RLX increased the peak I Na density by 2-fold without altering the characteristics of the current-to-voltage relationship (Figure 7). RLX did not alter I Na acutely requiring ≥24 hours to upregulate the current. Human iPS-CMs largely represent mature human ventricular myocytes that exhibit low levels of inwardly rectifying K+ current. 29,30 RLX (100 nmol/L) was also found to upregulate I Na.
density of guinea pig atrial myocytes in 24 to 72 hours (data not shown). The time needed to enhance $I_{\text{Na}}$ in cultured iPS-CMs is a strong indicator of a genomic upregulation of Nav1.5 that occurs independently of the antifibrotic effects of RLX and provides a compelling proof of concept that RLX may suppress AF in human hearts.

**Discussion**

The main findings are that SHR hearts have a higher susceptibility to AF triggered by a single premature impulse. SHR atria had a slower CV and higher levels of collagen deposition (ie, fibrosis). RLX treatment of SHR animals for 2 weeks significantly reversed fibrosis and hypertrophy, increased atrial CV, and suppressed AF.

**Atrial Fibrosis and AF**

Atrial fibrosis has been implicated in the pathogenesis of AF, but a direct link between fibrosis and AF has not been established. Atrial tissue fibrosis is nevertheless a most consistent finding in patients and animal models of AF. Atrial tissue fibrosis has been implicated in the pathogenesis of AF, but a direct link between fibrosis and AF has not been established. Atrial tissue fibrosis is nevertheless a most consistent finding in patients and animal models of AF. Our histological studies confirm that SHR hearts are fibrotic and hypertrophic compared with controls. In addition, SHR atria are characterized by conduction abnormalities that provide a basis for lines of conductional block that promote reentry as seen in optical mapping studies. The major mechanisms that have been proposed for the initiation and maintenance of AF are the multiple wavelet theory, focal activity hypothesis, and single-circuit reentrant theory. Our optical mapping studies were consistent with AF generated by coexisting reentrant circuits with varying origins, which supports the multiple wavelet theory as the mechanism of AF.

**Antifibrotic and Antiarrhythmic Properties of RLX and Its Clinical Relevance**

Relaxin mediates effects on the cardiovascular system by activating a wide range of signaling pathways via the relaxin family peptide receptor 1, a G-protein–coupled receptor that leads to an acute elevation of cAMP and nitric oxide. In other
studies, RLX has been shown to inhibit fibroblast proliferation, differentiation, collagen synthesis, collagen deposition, and increased MMP-2 expression, which most likely contributed to an increase in collagen degradation and a decrease in collagen deposition.36 Our results demonstrate the increased collagen I deposition, transcripts encoding the profibrotic cytokine TGF-β, the major ECM fibrotic component collagen I, and MMP-2 and -9 in SHR atria relative to normotensive WKY, similar to previous reports for SHR left ventricle and atrial tissues.17,37 We also observed a relaxin-induced decrease in atrial collagen I and collagen transcripts and TGF-β transcripts, similar to that reported for the SHR-LV and in a model of interstitial renal fibrosis,23,24,38 and consistent with a role for inhibition of TGF-β expression or signaling in the reversal of cardiac (and other organ) fibrosis by RLX.39 We observed a decrease in RNA encoding MMP-2 and MMP-9 in response to RLX treatment, whereas an increase in MMP-2 activity has been previously observed in SHR ventricles treated with RLX.24 However, our observations are consistent with reports that in atria from rats or dogs subjected to interventions that increase AF susceptibility, fibrosis and MMP expression/activity were both elevated40,41 and that MMP inhibition reversed atrial cardiomyocyte hypertrophy, MMP activity, collagen deposition, and AF inducibility.41 Clearly, a more complete mechanistic understanding of the reversal of atrial fibrosis by RLX will require a broader examination of the activity of multiple MMPs and of their endogenous inhibitors (tissue inhibitors of metalloproteinases).

Targeting fibrosis has been attempted with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and a novel compound pirfenidone. However, most of these studies have examined models of heart failure, which is less commonly associated with AF than hypertension. Pirfenidone has been shown to reverse fibrosis and attenuate AF in a congestive heart failure canine model.42 Pirfenidone treatment achieved reversal of atrial fibrosis and reduced vulnerability of AF after burst pacing but did not generate a significantly greater increase in atrial CV. In contrast, our data show that treatment with RLX reduces AF inducibility, reverses atrial fibrosis and hypertrophy, increases CV, and decreases APD. It is important to note that RLX treatment of SHRs for 1 week was ineffective in suppressing AF and that longer RLX treatment was necessary because remodeling of the ECM and gap junctions may be reversed, albeit slowly. Reversal of fibrosis is a slow process as a result of the slow collagen turnover rate of 5% per day in healthy hearts.43 Enhanced atrial fibrosis can in turn alter connexin 43 expression and its redistribution to lateral cell borders, creating a barrier to impulse propagation by reducing intermyocyte coupling and CV.4 However, it is difficult to evaluate the amount of connexin disruption that is required to produce...
a significant change of CV, and an alternative mechanism is to increase CV by an upregulation of \( I_{Na} \) density. The pleiotropic effects of RLX and its relevance to human hearts were demonstrated by testing its effects on cultured human iPS-CMs. Independent of fibrosis, RLX increased sodium current density in 48 hours, indicating that RLX acted at fibroblasts to remodel ECM and human myocytes to alter ion channel expression. The actions of 2 weeks of RLX treatment differ from the acute effects of RLX. In rat hearts, RLX was found to bind to atrial tissue, increase heart rate, prolong APD by inhibiting the \( I_{Na} \) K+ current, and increase Ca2+ influx because of APD prolongation. The acute effect of RLX on heart rate was readily measured in perfused hearts, but the longer-term effects of RLX on increased atrial CV and reduced APD relative to SHR+V controls imply additional direct effects on ion channel properties and expression, as well as its antiﬁbrrotic effects.

**Efficacy and Safety**

RLX has been under clinical trials for acute heart failure with a completed 234-patient phase 2 and an ongoing 160-patient phase 3. Reports have conﬁrmed the safety of RLX infusion in humans (≤0.96 mg/kg per day) and have noted a vasodilatory effect in patients with heart failure, but RLX therapy did not always improve renal functions. The clinical trials to date have sensibly addressed potential beneﬁts of short-term treatment in vasodilation, but have not examined whether other pathways mediated by RLX can be exploited to provide long-term therapeutic beneﬁts.

**Limitations**

Our studies used the SHR model that exhibits many parallels to human hypertension. However, ECM remodeling in hypertension and mechanisms of AF may differ in rats and humans. The exact mode of action of RLX at suppressing AF is complex; RLX has the anticipated antiﬁbrrotic effects on the atria, but reversal of fibrosis may not be sufﬁcient to explain the marked increase in CV, which is the predominant mechanism for AF suppression. As proof of concept that RLX modulates cardiac properties independent of ﬁbrosis and is relevant to human AF, we tested the effects of RLX on the voltage-gated sodium current in cultured human iPS-CMs. RLX treatment for 48 hours markedly upregulated \( I_{Na} \) density (from \( \sim 22.95±5.8 \) to \( \sim 38.64±10 \) pA/pF, mean±SEM) most likely by a genomic mechanism that could explain the increase in CV and faster AP rise time. Hence, longer-term treatment with RLX suppresses AF, in part, by reversing fibrosis, enhancing connexin 43 phosphorylation, and upregulating voltage-gated Na+ channels. Still other contributing factors cannot be excluded, and further studies will be needed to fully characterize the panelopy of the actions of RLX.

Possible adverse effects of RLX cannot be excluded, and the cardiovascular safety of RLX will have to be stringently tested before RLX can be a game-changing therapy for the treatment of AF.

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**References**


Novelty and Significance

What Is Known?

- Major risk factors for atrial fibrillation (AF) include sex (men > women), old age, hypertension, and enhanced tissue fibrosis.
- Fibrosis is thought to be arrhythmogenic, as it correlates with altered connexin expression, hypertrophy, and a slowing of conduction velocity.
- AF therapy is often ineffective; treatments with drugs and ablation are limited by intolerance, toxicity, and destruction of viable tissue.

What New Information Does This Article Contribute?

- Systemic administration of the pleiotropic hormone relaxin for 2 weeks suppresses AF inducibility in spontaneously hypertensive rat hearts.
- Relaxin acts by reversing fibrosis and myocyte hypertrophy in atrial tissue and increasing conduction velocity, and in cultured human myocytes derived from stem cells, relaxin increases the sodium current, which is consistent with the increase in conduction velocity.
- The effects of relaxin on rat atrial tissue and human myocytes are proof of concept for a new approach to remodel heart tissues and develop more effective therapy for AF patients.

AF is a serious public health problem in dire need of new solutions because current treatments involve either the destruction of viable tissue (ablation) or the risk of cardiac toxicity (drug therapies). In spontaneously hypertensive rats, sustained AF is readily elicited by a single premature impulse. We report here that a 2-week treatment with the reproductive hormone relaxin suppresses AF in spontaneously hypertensive rat hearts by reversing fibrosis and myocyte hypertrophy and causing a marked increase in conduction velocity. In models of hypertension and age-related AF, a slowing of conduction velocity has been associated with AF vulnerability. The possibility that relaxin increases conduction velocity by acting directly on myocytes was tested in cultured human cardiomyocytes derived from inducible pluripotent stem cells, where relaxin increased the magnitude of the voltage-gated Na⁺ current density, I₅ Na by 2-fold in 48 hours, which is consistent with the higher conduction velocity. These findings indicate that relaxin acts on the intracellular matrix through fibroblast modifications and directly on myocyte level. The antifibrotic and antihypertrophic actions of relaxin and its genomic upregulation of I₅ Na provide compelling evidence that relaxin may provide a novel and transformative therapy to treat AF.
Relaxin Suppresses Atrial Fibrillation by Reversing Fibrosis and Myocyte Hypertrophy and Increasing Conduction Velocity and Sodium Current in Spontaneously Hypertensive Rat Hearts

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Supplemental Material

Relaxin suppresses atrial fibrillation by increasing conduction velocity through a reversal of fibrosis and hypertrophy and increased sodium current in spontaneously hypertensive rat hearts

Supplemental Methods

Blood pressure, Heart Rate and Serum RLX Concentration

Heart rate, diastolic and systolic blood pressures (BP) were measured using a tail cuff (Coda 6, Kent Scientific Corp., Torrington, CT), at 3 time-points of the treatment: pre (day 0), mid- (day 7) and post-treatment (day 14). Serum RLX concentration was assayed using a commercial kit (Quantikine Human Relaxin-2 Immunoassay, R&D Systems, Minneapolis, MN, USA).

Optical Apparatus and Analysis

Rats were anesthetized with pentobarbital (50 mg/kg), injected with heparin (200 U/kg IV), then the heart was excised and perfused on a Langendorff apparatus with physiological Tyrode’s solution containing (in mM): 122 NaCl, 25 NaHCO3, 4.81 KCl, 2 CaCl2, 2.75 MgSO4, 5 Glucose (pH 7.4) gassed with 95 percent O2 and 5 percent CO2 at 37.0±0.2 °C.

Hearts were placed in a chamber and perfused with blebbistatin (3-5 µM) for 5-10 min to arrest contractions and reduce motion artifacts; if needed blebbistatin perfusion was repeated ~1-hour later. The hearts were stained with bolus injections of a voltage-sensitive dye (PGH-1; 300 µl of 1 mg/ml in dimethyl sulfoxide, DMSO) and a Ca2+ indicator (Rhod-2/AM, 300 µl of 1 mg/ml in DMSO), as previously described.

Light from a 100-W tungsten-halogen lamp was collimated, passed through 530 ± 30 nm interference filters, split by a 560 nm dichroic mirror and focused on the atria. Fluorescence from the stained heart was collected with tandem camera lenses (50 mm f/1.2 mm Nikon and 50 mm f/0.95 Navitar), was split with a 600 nm dichroic mirror to focus images of the atria at short (570-595 nm) and long (610-750 nm) wavelengths on two 100 x 100 pixel CMOS cameras (Ultima, Scimedia, Ltd. Tokyo, Japan). Each camera was scanned at 2,000 frames per second. Pixel resolution was 150 x 150 µm2, and the data was recorded and stored in intervals of 4-8 seconds.

Activation and repolarization time points at each site were determined from fluorescence (F) signals by calculating (dF/dt)max and (d2F/dτ2)max, which has been shown to coincide with ~97% repolarization to baseline and recovery from refractoriness.
Action potential duration was measured from \((dF/dr)_{\max}\) to 90% recovery to baseline, APD\(_{90}\). Mean APD\(_{90}\) was calculated for each heart by averaging APD\(_{90}\) from a region of atrium consisting of 10x10 pixels or 100 APD\(_{90}\) from each heart for a minimum of 5 hearts. Local conduction velocity (CV) vectors were calculated for each pixel from the differences in activation time-points of that pixel (determined from \((dF/dr)_{\max}\)) and its 7 × 7 nearest neighbors, as previously described.\(^3\) Local CVs were averaged and calculated as means ± standard deviation (SD). Local CV can be overestimated when two wave fronts collide, transmural propagation breaks through the surface, or when activation appears synchronous over a region of the atrium because of its proximity to the pacing electrode. To avoid overestimations of CVs, CVs > 1.25 m s\(^{-1}\) were deleted from the analysis.\(^4\) Time and frequency domains analysis was achieved, as previously described.\(^5\) APD Restitution Kinetics (RK) curves were generated by plotting mean APD\(_{90}\) (from a minimum of 100 pixels per atrium times a minimum of 5 hearts (right or left atria)) versus S1-S2 interval in milliseconds. CV RK curves were generated by plotting the mean CV from a minimum of 5 atria vs. S1-S2 interval in milliseconds.

**Programmed Stimulation**

To test AF vulnerability, each heart was paced at the RA using a programmed stimulation protocol consisting of 20 S1 pulses at 250 ms cycle length (CL) followed by a premature S2 pulse with progressively shorter S1-S2 interval steps: 250 to 100 ms in 20 ms steps; 100 to 60 ms in 10 ms steps and 60 to 35 in 5 ms steps, until loss of capture or the initiation of AF.

**Immunofluorescence Imaging**

Atrial tissues were fixed in 2% paraformaldehyde, equilibrated in 30% sucrose, and flash frozen in supercooled isopentane. Frozen sections (7 micron thick) were cut by cryostat and sections interacted with rabbit anti-mouse collagen I (1:1000 dilution, Chemicon #AB765), Hoechst 33342 (1:1000 dilution, to identify nuclei, Sigma), and phalloidin 488 (1:250 dilution, Alexa Fluor 488 phalloidin A12379, Invitrogen, to identify filamentous actin). Fluorescent secondary antibodies included goat anti-rabbit IgG conjugated with Cy3 (1:1000 dilution, Molecular Probes #A10520, Invitrogen). To assess the severity of cardiac hypertrophy, left atrial cardiomyocytes were mounted on slides which were stained with Alexa-488-labelled wheat germ agglutinin (1:1000 dilution; Invitrogen, #W11261) to measure the cross sectional area of the cardiomyocytes, as previously described.\(^6\) For statistical significance, 10 myocytes were randomly selected from each of 5-10 sections of tissue from each group of rat hearts. Slides were viewed at 20X with a fluorescent microscope (Olympus Provis). Images from different wavelengths were collected with a cooled CCD camera at 24-bit gray depth and assembled (Adobe Photoshop). Collagen I to tissue area ratio was calculated as previously described using the area stained with phalloidin to index tissue area, averaging 6 random fields per heart, with 3-5 rats per group,\(^7\) and analyzing right and left atria separately.

**Analysis of connexin 43 phosphorylation**
The relative phosphorylation status of connexin 43 in right atria of SHRs treated with a vehicle (n=4) or relaxin (n=4) was measured using Western blots. We focused on connexin 43 because previous reports failed to detect connexin 40 in rat atria. Frozen right atria from SHR animals were homogenized in RIPA buffer (Thermo Cat#89900), containing protease (Cat#P8340 Sigma-Aldrich) and phosphatase inhibitors (Cat#5726 Sigma-Aldrich), and briefly centrifuged to remove gross debris. Protein levels were determined by Protein Assay (Bio-Rad) using bovine IgG as a standard. Proteins (25 µg/sample) were subjected to 12% polyacrylamide gel electrophoresis, transferred to PVDF membranes, interacted with antibody (Rabbit anti-Connexin 43, Invitrogen Cat#71-0700), washed, and developed by chemiluminescence. Digitized films were analyzed using NIH Image J to determine the ratio of the phosphorylated (43 kD) to non-phosphorylated (40 kD) connexin 43.

**RT-PCR Analysis**

RNA was isolated (RNAEasy, Qiagen) and copied to cDNA (High Capacity Reverse Transcription kit, Applied Biosystems) according to manufacturer protocols. A Syber-green-based formulation (Absolute Sybr-Green, Thermo Fisher Scientific, Waltham, MA) was utilized for fluorescence-based kinetic real-time PCR using an Applied Biosystems model 7000 detection system (Applied Biosystems Inc., Foster City, CA). Expression levels of RNAs of interest were normalized to that of GAPDH using the Ct method, and reported relative to the mean of the WTV group. Primer pair sequences (forward and reverse for each target, listed 5’ to 3’) used for RT-PCR are as follows; MMP-2: gcaccacgaggattagac, cacccacagtggacatagca; MMP-9: cctctgcatgaagacgacataa, ggtcaggtttagagccacga; Collagen I: catgtccagcttggtgact, gcacgtgcatcgggtgt; Collagen III: tccctggcatctgtagaatc, tgatggatgagggagaat; TGF: cctggaaaggctcaaac, cagttcttctgtggagctga; GAPDH: agctggtcatcaatgggaa, atttgatgtagccgggtac.

**Statistics**

AF vulnerability between the different groups was compared using Fisher’s exact test. Parameters recorded under different S1-S2 were compared using ANCOVA. For RT-PCR, western blot, and immune-fluorescence microscopy, comparisons among three or more groups were performed using a non-parametric test (Kruskal-Wallis) with post-hoc analyses (Conover). All results are reported as mean ± SD unless otherwise stated. For all tests, a value of \( p < 0.05 \) was considered to be statistically significant.

**Cardiomyocyte (CM) differentiation of human iPS cells: iPS-CMs**

A human Y1 iPS cell line was generated in L.Y’s lab from human fibroblast line HDF-α as previously described. The following conditions were used for cardiomyocyte differentiation using the basal StemPro®-34 (Invitrogen) medium as described in our previous study: days 0–1, BMP4 (10 ng/ml); days 1–4, BMP4 (10 ng/ml), bFGF (5 ng/ml) and Activin A (1.5 ng/ml); days 4–
8, DKK1 (150 ng/ml) and VEGF (10 ng/ml); after day 8, VEGF (10 ng/ml), bFGF (10 ng/ml), BMP4 (1ng/ml) and DKK1 (150 ng/ml).

 Cultures were maintained in a 5% CO₂/5% O₂/90% N₂ environment for the first 20-days and were then transferred into a 5% CO₂/air environment. All cytokines were purchased from R&D Systems.

Cell Culture and RLX Treatment

Human iPS-CMs were seeded on 15 mm cover-slips coated with 0.01% (w/v) gelatin solution placed in 12-well plates. Cells are seeded at a density of 20,000-40,000 viable iPS-CMs per a dish in 2 mL of room temperature plating medium, permitting the cells to culture as single cells. Cells were incubated for at least 2 days at 37°C, 7% CO₂. Non-adherent cells are removed after 2 days by rinsing with basal differentiation medium. Plated iPS-CMs are maintained by changing the 2 mL of maintenance medium every 2 days. Relaxin treated coverslips contained 0.1 µM of Recombinant Human Relaxin (supplied by Corthera-Novartis (Basel, CH) for 48 hours prior to voltage-clamp experiments.

Voltage-Clamp Protocols

Cardiac action potentials and ionic currents were recorded from single iPSC derived myocytes. Ionic currents were recorded using the whole-cell patch clamp technique performed at room temperature using Axopatch1D, Digidata 1322A, and pClamp 9 (Axon Instruments) for data amplification, acquisition and analysis. Cells in Tyrode solution were kept in a recording chamber (300 µl volume) and were continuously perfused with fresh Tyrode solution. Suction pipettes, were fabricated from borosilicate glass using a Flaming/Brown horizontal micropipette puller with resistances between 2 and 4 MΩ. Action potentials were recorded in the current clamp mode and sodium current magnitudes were measured as the rapid peak inward current recorded in the same solution under voltage clamp mode. APs were elicited by a current injection through the patch, sufficient to elicit an upstroke. Patch pipettes contained the following intracellular solution (mM): 140 KCl, 1 MgCl₂, 5 EGTA, 5 ATP (Mg salt), 5 Na₂-creatinephosphate, 0.2 GTP, and 10 HEPES, pH 7.4 and extracellular solution contained (mM): 144 NaCl, 5.4 KCl, 1 MgCl₂, 2.5 CaCl₂, 5.6 glucose, and 10 HEPES, pH 7.4. Currents were elicited by a protocol of depolarizing potentials of -130 mV to 50 mV in 10 mV increments from a holding potential of -80 mV. Current densities were measured as the peak current for each potential pulse. Currents were normalized to the cell capacitance and expressed in pA/pF.
**Supplemental Data**

**Table I: Effect of RLX treatment on blood pressure (BP) and heart rate (HR)**

<table>
<thead>
<tr>
<th></th>
<th>Pre-Tx</th>
<th>Mid-Tx</th>
<th>Post-Tx</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tail BP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR+RLX</td>
<td>156 ± 20 (14)</td>
<td>155 ± 24 (7)</td>
<td>173 ±14 (6)*</td>
</tr>
<tr>
<td>SHR+V</td>
<td>157 ± 34 (5)</td>
<td>165 ± 12 (3)</td>
<td>164 ± 27 (4)</td>
</tr>
<tr>
<td><strong>HR (BPM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR+RLX</td>
<td>427 ± 18 (10)</td>
<td>465 ± 43 (7)*</td>
<td>478 ± 27 (6)*</td>
</tr>
<tr>
<td>SHR+V</td>
<td>393 ± 48 (5)</td>
<td>400 ± 78 (4)</td>
<td>430 ± 63 (4)</td>
</tr>
</tbody>
</table>

Blood pressure (BP) and heart rate (HR) were measured before RLX treatment (Pre-Tx), midway or 1-week after RLX treatment (Mid-Tx) and after 2-weeks of RLX treatment (Post-Tx). Mean values are given ± S.D, number of rats for each group is shown in parentheses; * versus Pre-TX (SHR+RLX) $p < 0.05$
Table II: In right Atria, effect of RLX on APD90, CV and AP Rise-time of vs. cycle length (CL)

<table>
<thead>
<tr>
<th>CL</th>
<th>APD90</th>
<th>CV</th>
<th>Rise Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY (n=5)</td>
<td>SHR (n=5)</td>
<td>SHR+V (n=4)</td>
</tr>
<tr>
<td>250</td>
<td>98±14</td>
<td>89±15</td>
<td>68±7</td>
</tr>
<tr>
<td>200</td>
<td>92±12</td>
<td>81±13</td>
<td>59±7</td>
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<tr>
<td>180</td>
<td>89±11</td>
<td>81±17</td>
<td>55±6</td>
</tr>
<tr>
<td>160</td>
<td>86±11</td>
<td>79±14</td>
<td>57±6</td>
</tr>
<tr>
<td>140</td>
<td>80±10</td>
<td>78±10</td>
<td>56±5</td>
</tr>
<tr>
<td>120</td>
<td>76±7</td>
<td>72±6</td>
<td>56±4</td>
</tr>
<tr>
<td>100</td>
<td>66±3</td>
<td>66±5</td>
<td>53±4</td>
</tr>
<tr>
<td>90</td>
<td>63±3</td>
<td>63±3</td>
<td>52±1.6</td>
</tr>
</tbody>
</table>

Similar findings were obtained in RA compared to LA (shown in Table 1). Rat hearts were perfused in a Langendorff apparatus and paced on the RA while mapping optical action potentials (AP) from the RA, field-of-view was 3x3 mm². AP durations (APD), conduction velocity (CV) and the rise-time of AP upstrokes were measured as a function of cycle length in ms. For WKY, SHR and SHR+RLX, n= 5 heart per group, for SHR + V treatment n= 4 hearts. *p<0.05 vs. SHR; †p<0.05 vs. WKY, SHR and SHR+V, (ANCOVA)
Figure I: Effects of RLX treatment on RLX in Blood Serum and AP characteristics

A: Blood serum concentrations of recombinant RLX were measured pre and post treatment in SHR implanted with mini-pumps containing either RLX or V. RLX was not detected in any of the rats unless treated with RLX. Histograms shown as mean [RLX] ± SEM

B: Illustration of AP recorded from the LA of WKY and SHR hearts without and with 2-weeks of RLX treatment.
Figure II: Role of Ca$_i$ in AF inducibility

Superposition of AP (blue) and Ca$_i$T (red) from LA of SHR during (A) S1-S2 = 60 ms; (B) during the initiation of sustained AF at S1-S2 = 55 ms.
Figure IIIA: In SHR+V hearts, a single premature impulse at S1-S2 = 50 ms elicits AF that is sustained and does not stop spontaneously (lower trace). Panels 1-9: Activation maps from an SHR+V heart from 9 consecutive beats labeled 1-9 in the trace to depict the last 3 normal beats and the transition beats to AF panels 4-6 and 3 beats during AF panels 7-9. Note that in untreated SHR hearts the first spontaneous beat propagates at a slower CV (panel 6) compared to SHR+RLX panel 8 in Figure 2SB. Isochronal lines are 1 ms apart.
**Figure IIIIB.** In SHR+RLX hearts, a single premature impulse at S1-S2 = 35 ms elicits a brief transient tachycardia that self-terminates after one extra beat. Panels 1-5 show the activation maps of paced beats and panel 6 shows the activation that is interrupted by a premature impulse panel 7. The last beat propagates rapidly and self-terminates. Isochronal lines are 1 ms apart.
References


