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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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Abstract

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 as a plausible therapy.

Objective—To evaluate the efficacy of the anti-fibrotic hormone relaxin (RLX) at 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 (0.4mg/kg/day, SHR+RLX) using implantable mini-pumps. Hearts were perfused, mapped optically to analyze action potential durations (APDs), intracellular Ca^{2+}-transients, restitution kinetics (RK) and tested for AF vulnerability. SHR hearts had slower conduction velocity (CV) ($p< 0.01$ vs. WKY), steeper CV RKs, greater collagen deposition, higher levels of transcripts for TGFβ, metalloproteinase-2, metalloproteinase-9, collagen I/III and reduced connexin-43 phosphorylation ($p< 0.05$ vs. WKY). Programmed stimulation triggered sustained AF in SHR (n=5/5), SHR+V (n=4/4) but not in WKY

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DISCLOSURES
None

(n=0/5) and SHR+RLX (n=1/8, $p<0.01$). RLX treatment reversed the transcripts for fibrosis, flattened CV-RK, reduced APD_{90}, increased CV ($p<0.01$) and reversed atrial hypertrophy ($p<0.05$). Independent of anti-fibrotic actions, RLX (0.1μM) increased Na^+-current density, I_{Na} (~2-fold in 48-hours) in human cardiomyocytes derived from iPSCs (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 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 modulating cardiac ionic currents.

Keywords

Relaxin; atrial fibrillation; fibrosis; hypertrophy; I_{Na} upregulation; optical mapping; spontaneously hypertensiver rats

INTRODUCTION

Atrial Fibrillation (AF), a disease associated with mortality, morbidity and high costs, affects tens of millions of people worldwide and is increasing in prevalence.[1, 2] Among the many risk factors that promote the development of AF, the most prominent are sex (more prevalent in males than females), old age (>60 years-old) and hypertension.[2] Hypertension and aging lead to structural changes of the extracellular matrix (ECM) and enhanced AF vulnerability due to the altered myocardial substrate.[3] Another etiology of AF is atrial tachycardia which leads to electrical remodeling and altered intracellular Ca^{2+} homeostasis associated with decreases in action potential duration (APD) and shortened atrial refractory periods.[4, 5] Fibrosis is a hallmark of arrhythmogenic ECM remodeling,[6] occurs with alterations in connexin expression, and slows conduction velocity (CV), creating a barrier to impulse propagation by disrupting inter-myocyte coupling.[6,7]

Increased collagen deposition has been well documented in AF patients compared with control subjects.[8] Although the precise signaling processes of fibrosis are unknown multiple factors have been implicated (e.g. angiotensin II (AII), Transforming Growth Factor β (TGF-β1) and Platelet Derived Growth Factor (PDGF)) in the pathogenesis of atrial fibrosis. ACE overexpression is associated with atrial enlargement, atrial fibrosis, and AF,[9] whereas blockade of ACE blunts atrial fibrosis and AF in animal models and patients with HF.[9] TGF-β1 and PDGF are thought to act on cardiac fibroblasts to increase collagen production without offsetting increases in collagen degradation.[5] It should be noted that the role of fibrosis as the cause of AF can be overstated since some studies show no difference in fibrosis in AF and control patients.[10] A possible explanation that remains unproven is that only some forms of collagen deposition cause AF; namely interstitial[11] and/or disorganized[12] 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/or 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 better and safer drug therapies for the treatment of AF.[13, 14] Existing anti-arrhythmic drug approaches have limited effectiveness and are associated with risks of serious complications, particularly ventricular pro-arrhythmia and/or organ toxicity.[2]

The Spontaneously Hypertensive Rat (SHR) has been widely studied as model of the effects of hypertension on the cardiovascular system.[15] 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.[16] Previous studies on the SHR model have shown an

increased incidence of AF and atrial tachyarrhythmias compared to normotensive Wystar-Kyoto (WKY) rats, attributed to greater levels of fibrosis.[17] These findings suggest that fibrosis may promote the development of AF making it an important anti-arrhythmic target.

Relaxin (RLX), a pleiotropic hormone, which is widely conserved, has been shown to have a wide range of biological actions including anti-inflammatory, anti-apoptotic, cardioprotective, vasodilatory, pro-angiogenic effects, and anti-fibrotic effects.[18, 19] RLX was first identified for its role in reproduction and pregnancy. It is thought to play a critical role in the hemodynamic adaptive and anti-fibrotic changes that occur during pregnancy.[19–21] 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.[22]

In the present report, we demonstrate that exogenous systemic administration of RLX to spontaneously hypertensive rats suppresses AF inducibility by reversing fibrosis and hypertrophy, and increasing CV. These actions of RLX may be relevant to human AF and as a proof-of-concept, we show that RLX upregulates I_{Na} in human 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 NIH (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 (9–12 months) and sex (male) matched rats (Charles River Laboratories) that were separated in four groups: 1) normotensive Wystar-Kyoto untreated rats (WKY); 2) untreated spontaneously hypertensive rats (SHR); 3) SHR treated with the vehicle saline (SHR+V); 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/day (for 400 g rats) over the 14-day period. This dose of RLX is comparable to the dose previously used to treat *in vivo* rodent models of fibrosis[23–25] and to examine RLX’s effects on arterial hemodynamics and vascular mechanical properties.[25, 26] Pumps were surgically implanted under sterile technique into the subcutaneous space on the left side of anesthetized animals. Animals were monitored over 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 two groups were combined in some figures which also allowed us to display the findings more clearly. For western blot and RT-PCR analysis, the four 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: pre (day 0), mid- (day 7) and post-treatment (day 14), as described in the supplement. Hearts were perfused in a Langendorff apparatus to simultaneously map action potentials (APs) and intracellular Ca^{2+} transients (CaTs) using standard techniques (see 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 (CL) followed by a premature S2 pulse (see supplement). Maps of APs were used to calculate conduction velocity (CV), generate activation maps, measure APD_{90} and investigate the nature of atrial fibrillation by time and frequency domain analysis using previously reported techniques (see supplement).

Transient AF lasted < 3s and self-terminated whereas sustained AF lasted > 3min and was terminated by a bolus injection of KCl (1M) 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 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 supplement for: MMP-2, Collagen I, Collagen III, TGFβ and GAPDH.

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.

RESULTS

AF vulnerability

AF was inducible in each of 5 SHR animals, but none of 5 WKY animals ($p<0.01$, Figure 1). In WKY hearts, a premature impulse close to the refractory period (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 1A: a, b). In SHR hearts (Figure 1B), a premature impulse at S1–S2 = 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, $p< 0.01$ vs WKY). In left atria while pacing at 250 ms CL, refractory periods (RP) were shorter than mean APD_{90} (WKY: RP= 40±13ms, APD_{90}=98±18 ms, n=5, p<0.05; SHR: RP=58±10ms, mean APD_{90}= 87±18ms, n=5, p<0.05). RPs were shorter in WKY vs. SHR atria (n=5 each, p<0.01) and in SHR hearts, sustained AF was initiated at S1–S2=70±12ms which was not significantly different than their mean RP (n=5, p=NS).

Optical mapping of atrial fibrillation

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: a'–g'), the origins of successive reentrant waves varied randomly and the arrhythmia was perpetuated by co-existing 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 co-existing reentrant circuits at different frequencies (9–20 Hz) and

energy densities (Figure 2C). The analysis showed that the right (RA) and left atria (LA) had similar dominant frequencies (13.7 ± 1.4 and 14.2 ± 0.8 Hz) (Figure 2D). In SHR hearts, abnormalities in Ca^{2+} homeostasis (e.g. larger Ca_iTs and spark amplitudes, normal L-type Ca^{2+} current density, $I_{Ca,L}$, and absence of heart failure) has been attributed to cellular hypertrophy resulting in altered coupling between Ca^{2+}-entry via $I_{Ca,L}$ and SR Ca^{2+}-release.[28] The altered SR Ca^{2+} release in SHR hearts suggested a potential mechanism to initiate and/or sustain AF, which we tested by simultaneous mapping of APs and Ca_iT to search for spontaneous (non-voltage dependent) Ca^{2+}-release and Ca_i oscillations. As shown in the supplement (Figure IIA) Ca_i followed V_m, during transient arrhythmia and sustained AF (Figure IIB); neither did Ca_i oscillations occur that were not associated by 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 figure 1S). Blood pressures were comparable between SHR+RLX and SHR+V animals at all-time points, indicating that RLX did not reverse the hypertension (see supplementary Table I).

RLX is known to cause an acute increase in heart rate, mediated by cAMP elevation consistent with our findings that RLX (100 nM) perfusion increased heart rate by 10–15% within a minute (n=5 per group: SHR or WKY). A similar 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 (RT) on the left atria of SHR hearts were measured as a function of CL and compared to values measured in untreated SHR and WKY hearts. These electrical characteristics are shown for the left atria in Tale 1, while the heart was paced on the right atria. APD_{90} were shorter in SHR than WKY ($p< 0.05$), shorter in and 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 WKY, SHR and SHR+V ($p< 0.005$, Table 1). AP rise-times tended to shorter in SHR than WKY hearts and SHR+RLX tended to further reduce rise times but these changes did not reach statistical significance. Similar results were obtained in the right atria while pacing on the RA, supplementary Table II. The shape and time course of APs from WKY, WKY+RLX, SHR and SHR+RLX hearts are illustrated in supplementary Figure IB.

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, one heart had an infarct) (Figure 3 A–B). In contrast, V treatment of SHR failed to suppress AF inducibility (n = 4/4; $p< 0.01$ vs. SHR+RLX) (Figure 3 C and D). 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 right or left atria, failed to elicit AF. In rare cases, the S2 impulse produced a non-sustained arrhythmia of < 10 beats (Figure 3A).

The mean RFs for SHR+V (51±4.3ms, n=4) and SHR+RLX (50±10ms, n=5) left atria 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 RK of CV of LA and RA compared to 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 APD_{90} RK curves compared to SHR+V and WKY hearts consistent with APD_{90} in Table 1. 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 (Supplemental figure III A&B, respectively). The slower CV of the premature pulse and of the first spontaneous reentrant beat in SHR+V atria helps to sustain AF.

Histological 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 to 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 since SHR+RLX had a significantly lower collagen/tissue ratio when compared to SHR and SHR+V ($p< 0.05$). SHR+V left atrial (LA) cardiomyocytes had a significant level of hypertrophy with greater cross-sectional area of LA myocytes (CSA = 146.9±07.2 μm^2) compared to WKY+V (95.5±10.6 μm^2, $p<0.01$). The CSA of WKY+RLX atrial myocytes (96.9 ±3.3 μm^2) did not differ from that of WKY+V. However, the CSA of LA cardiomyocytes from SHR+RLX was significantly less (100.8±2.98 μm^2, $p<0.05$) than that of SHR+V and not significantly different from either WKY group. Thus, RLX appeared to reverse atrial myocyte hypertrophy in SHR hearts.

Effect of RLX on Cx 43 phosphorylation andfFibrosis-related transcripts

The effect of RLX treatment on the relative phosphorylation of connexin 43 in SHR right atria was assessed by Western blot analysis, using the differential molecular weight of phosphorylated (43 kD) to non-phosphorylated connexin 43 (40 kD). Proteins from Relaxin-treated SHR showed a significantly greater ratio in the band intensity of the 43 to 40 kD 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 left atria (LA) of 4–5 rats per group (WKY+V, WKY +RLX, SHR+V, SHR+RLX) (Figure 6). TGFβ, MMP-2, MMP-9, collagen I, and collagen III transcripts were all significantly elevated in SHR+V versus WKY+V ($p< 0.05$ or less). In WKY, RLX-treatment did not alter fibrosis-related transcripts (Figure 6). In contrast, RLX-treatment significantly reduced all the transcripts except for collagen III, which exhibited a marked trend towards a decrease. For TGFβ, MMP-2, and MMP-9, transcripts levels in SHR +RLX were not different from their levels 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/or altered expression, localization and/or 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 cardiomyocytes derived from inducible pluripotent stem cells (iPS-CMs). Human iPS-CMs were cultured with vehicle or 0.1 μM RLX for 48 hours then I_{Na} density was measured using the whole-cell voltage-clamp technique (see methods in 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 at least 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 nM) was also found to upregulate I_{Na} density of guinea pig atrial myocytes in 24–72 hours (data not shown). The time needed to enhance I_{Na} in cultured iPS-CMs is a strong indicator of a genomic upregulation of Nav1.5 that occurs independently of the anti-fibrotic 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 (i.e. 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.[31] Our histological studies confirm that SHR hearts are fibrotic and hypertrophic compared to controls. In addition, SHR atria are characterized by conduction abnormalities that provide a basis for lines of conductional block that promote re-entry 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[32], focal activity hypothesis[33] and single circuit reentrant theory.[34] Our optical mapping studies were consistent with AF generated by co-existing reentrant circuits with varying origins which supports the multiple-wavelet theory as the mechanism of AF.

Anti-fibrotic and anti-arrhythmic 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 (RXFP1), a G-protein coupled receptor that leads to an acute elevation of cyclic AMP (cAMP) and nitric oxide (NO).[19, 35] In other studies, RLX has been shown to inhibit fibroblast proliferation, differentiation, collagen synthesis, collagen deposition and increase 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 pro-fibrotic cytokine TGFβ, the major extracellular matrix fibrotic component collagen I, and MMP-2 and -9 in SHR atria relative to normotensive WKY, similar to previous reports for SHR LV and/or atrial tissues.[17, 37] We also observed a relaxin-induced decrease in atrial collagen I and collagen transcripts, and TGFβ 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 TGFβ 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 elevated,[40, 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 (TIMPs).

Targeting fibrosis has been attempted with ACE inhibitors, ARBs, 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 CHF 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 shows that treatment with RLX reduces AF inducibility, reverses atrial fibrosis and hypertrophy, increases CV and decreases action potential duration (APD).

It is important to note that RLX treatment of SHRs for 1-week was ineffective at suppressing AF and that longer RLX-treatment was necessary because remodeling of the ECM and/or gap-junctions may be reversed, albeit slowly. Reversal of fibrosis is a slow process due to 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 inter-myocyte coupling and CV.[6] 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 was 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,[44] increase heart rate,[45] prolong APD by inhibiting the $I_{t.o}$ K^+ current[46] and increase Ca^{2+} influx due to APD prolongation.[47] 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_{90} relative to SHR+V controls imply additional direct effects on ion channel properties and/or expression as well as its anti-fibrotic 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.[48] Reports have confirmed the safety of RLX-infusion in humans (up to 0.96 mg//kg)/day) and have noted a vasodilatory effect in patients with HF, but RLX therapy did not always improve renal functions.[49] 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 which exhibits many parallels to human hypertension.[50] 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 anti-fibrotic 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 −22.95 ±5.8 to −38.64±10 pA/pF, mean ± SEM) most likely by a genomic mechanism which 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 panoply of RLX's actions. Possible adverse effects of RLX cannot be

excluded and the cardiovascular safety of RLX will have to be stringently tested before RLX can a game-changing therapy for the treatment of AF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

RLX	relaxin
APD	action potential duration
RK	restitution kinetics
CV	conduction velocity
CMs	cardiomyocytes
iPSCs	inducible pluripotent stem cells
iPS-CMs	CMs derived from iPSCs
MMP	metalloproteinase
TFD	time frequency domain
Ca_i	Intracellular free Ca^{2+}
V_m	Membrane potential

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Novelty and Significance

What Is Known?

- Major risk factors for atrial fibrillation (AF), include sex (males>females), 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 as 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, I_{Na} by 2-fold in 48 hours which is consistent with the higher CV. These findings indicate that Relaxin acts on the extracellular matrix through fibroblast modifications and directly on myocytes level. The anti-fibrotic, anti-hypertrophic actions of Relaxin and its genomic upregulation of I_{Na} provide compelling evidence that Relaxin may become a novel therapy to treat AF.

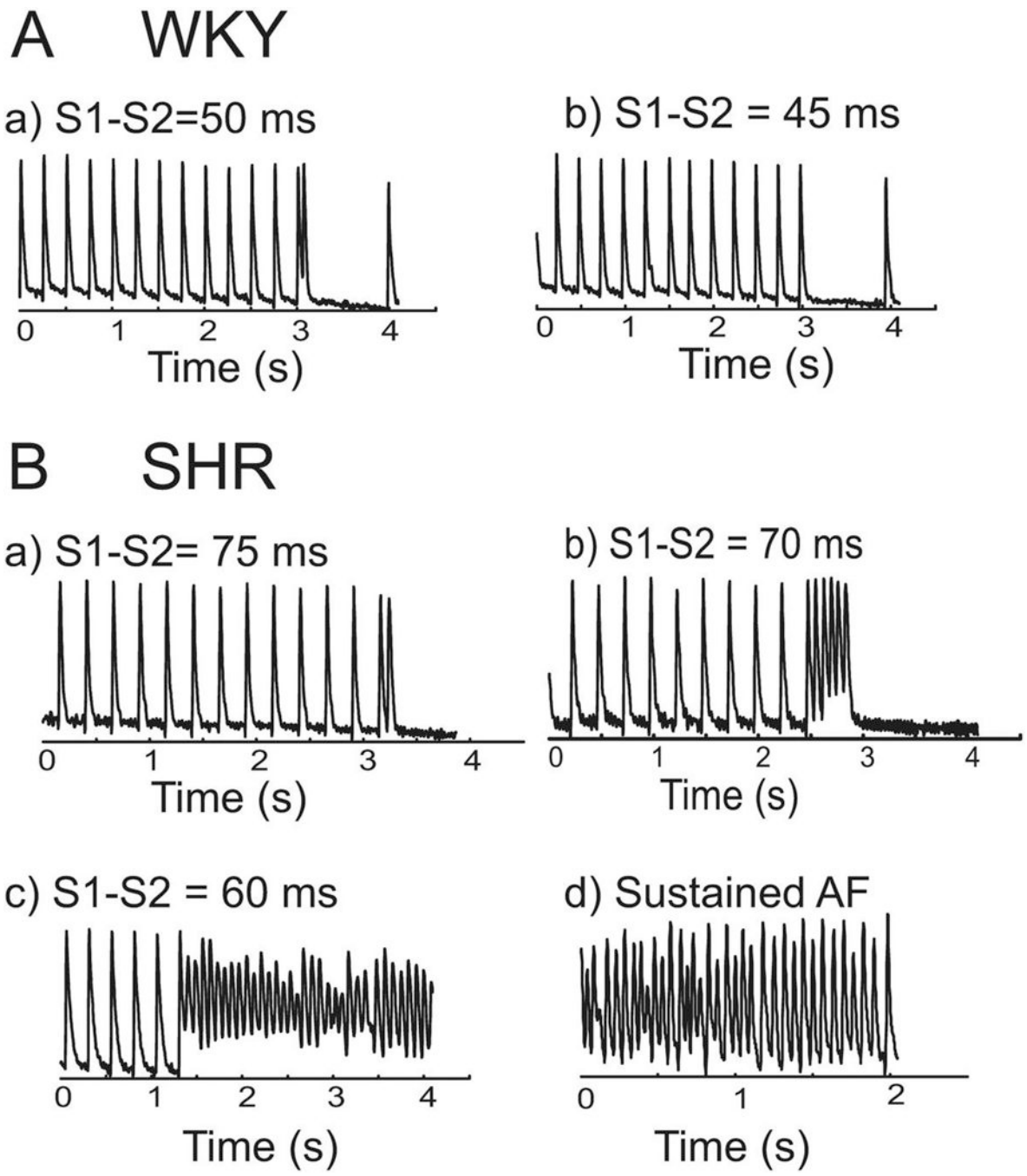

Figure 1. Inducibility of AF in normotensive and hypertensive rats

A: Representative action potential (AP) traces from the LA in normotensive WKY rat. As expected a premature pulse with an S1–S2 interval of 50 ms is longer than the refractory period and captures (a) whereas S1–S2= 45 ms is shorter than the refractory period and does not capture. No arrhythmias could be elicited by programmed stimulation or burst pacing. **B**: Representative AP traces from LA of SHR rat. With a premature pulse at S1–S2 = 75 ms, a normal AP captures and propagates (a); a impulse delivered at a shorter interval of 70 ms elicits transient arrhythmia and when delivered at still shorter interval of 60 ms, sustained AF are induced (c) which persisted for the duration of the experiment (d).

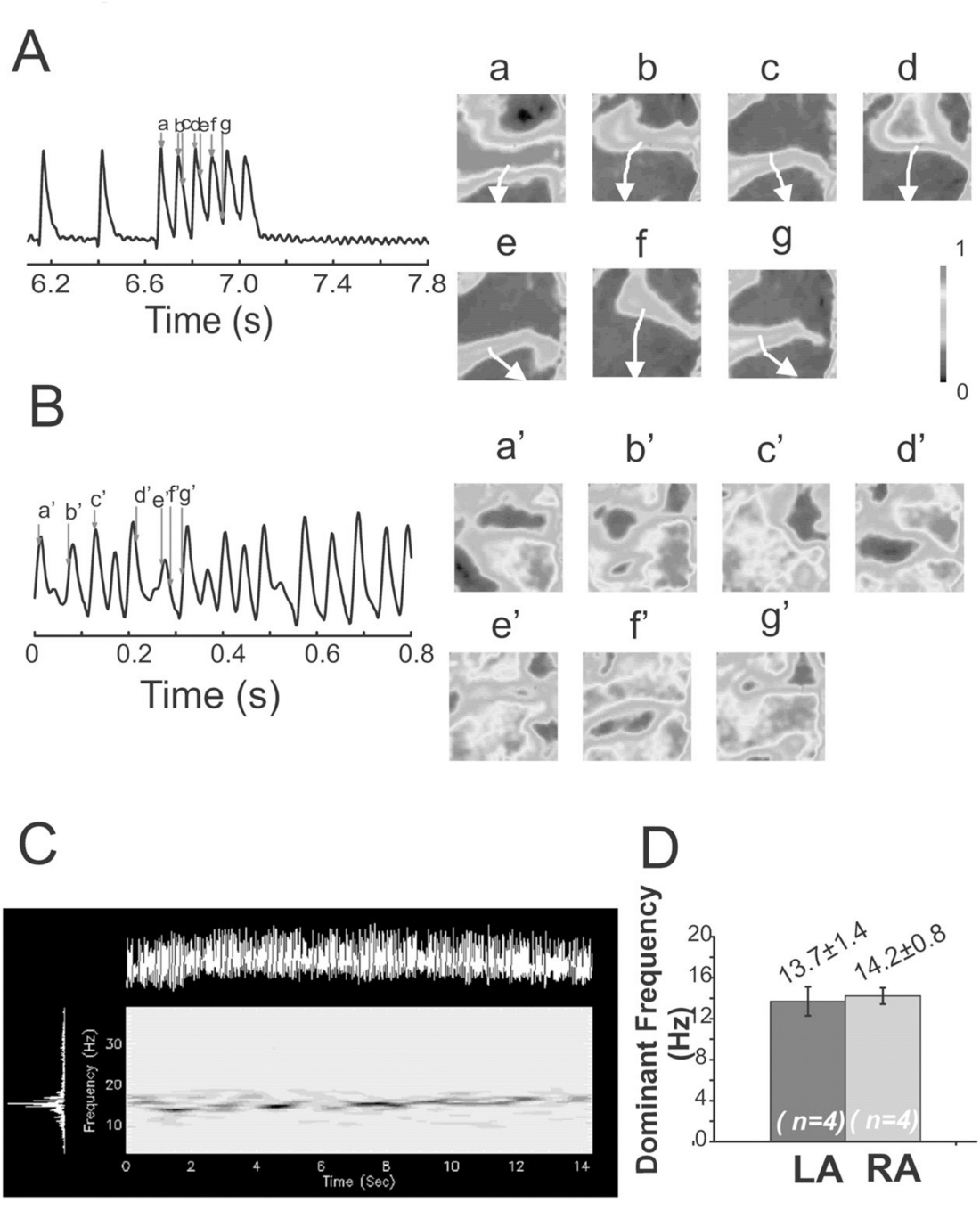

Figure 2. Analysis of the AF

A: Activation pattern on a 100×100 pixel CMOS with spatial resolution of 150×150 μm^2 exhibiting a single reentrant circuit during non-sustained AF. **B**: Activation pattern illustrating the creation and annihilation of multiple daughter waves (wavebreaks) during sustained AF. **C**: Time-frequency analysis of AF. The spectrogram was generated for each pixel by calculating the FFT spectrum for a brief Gaussian window of 2 seconds then shifting the window step-wise in time (Δt= 1 ms) and re-measuring the FFT spectrum at successive t intervals. Top, Optical trace; Left, Overall FFT spectra; Contour map, spectrogram with isolines drawn every 12.5% of maximum. Spectrogram plots frequency

(ordinate) versus time (abscissa) and is shown for 14 seconds of AF; the darker the color, the higher the energy density at that frequency. **D**: Histogram represents the dominant frequencies during sustained AF in SHR rats in the LA and the RA.

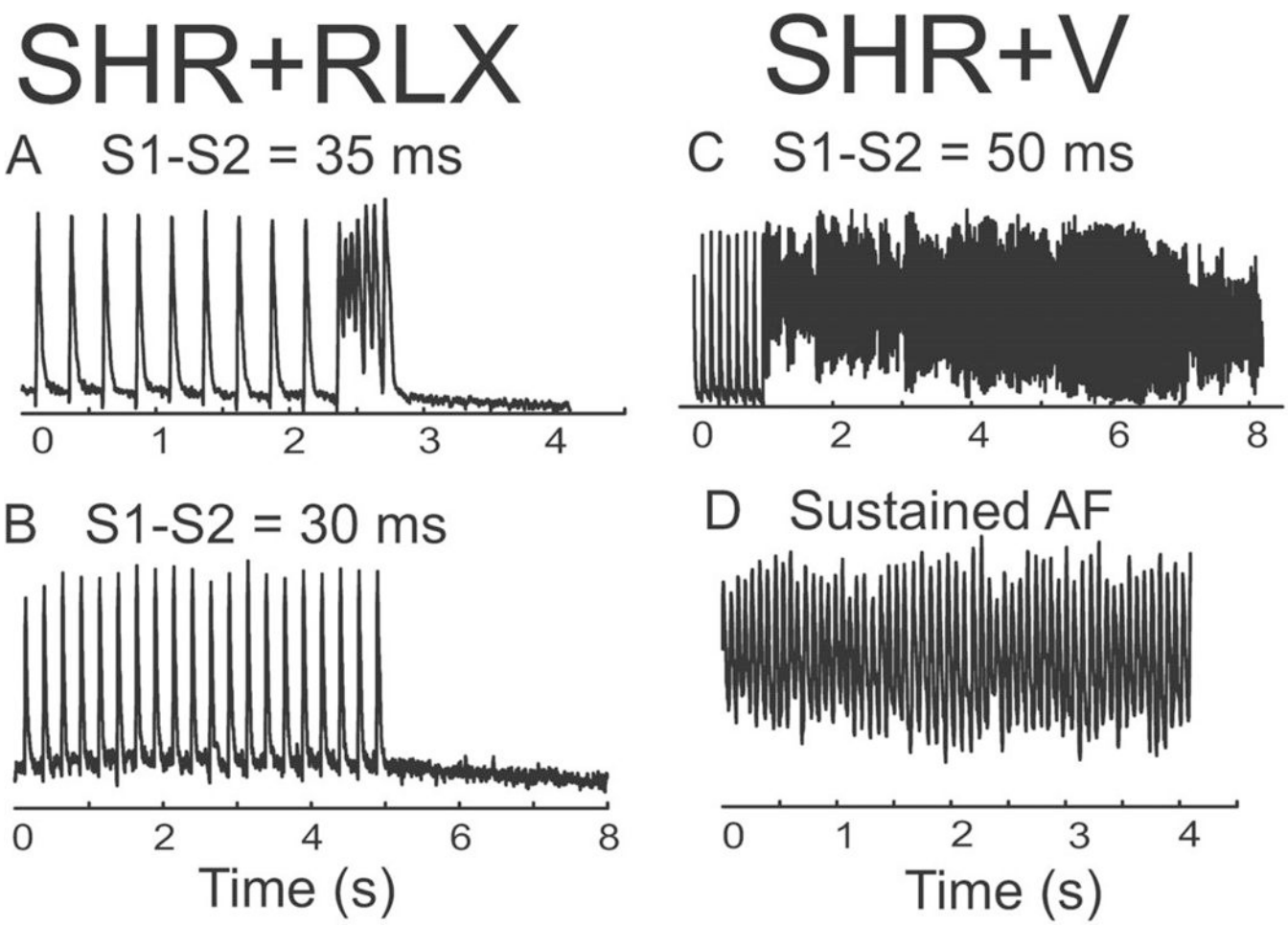

Figure 3. Effect of RLX on AF inducibility in SHR

Example of voltage (V_m) traces and activation maps from the LA of SHR+RLX (A to D) and from SHR+V hearts (E to H). **A**: An example of a non-sustained AF initiated by a single premature pulse using a short delay, S1–S2 = 35 ms. **B**: In most SHR+RLX hearts, no arrhythmias were elicited by a premature impulse and at S1–S2 = 30 ms the premature impulse failed to capture (n = 7/8). Activation maps from an SHR+RLX heart at 250 ms (**C**) and 90 ms (D) S1–S2 interval, note the rapid propagation of the premature impulse in panel **D**.

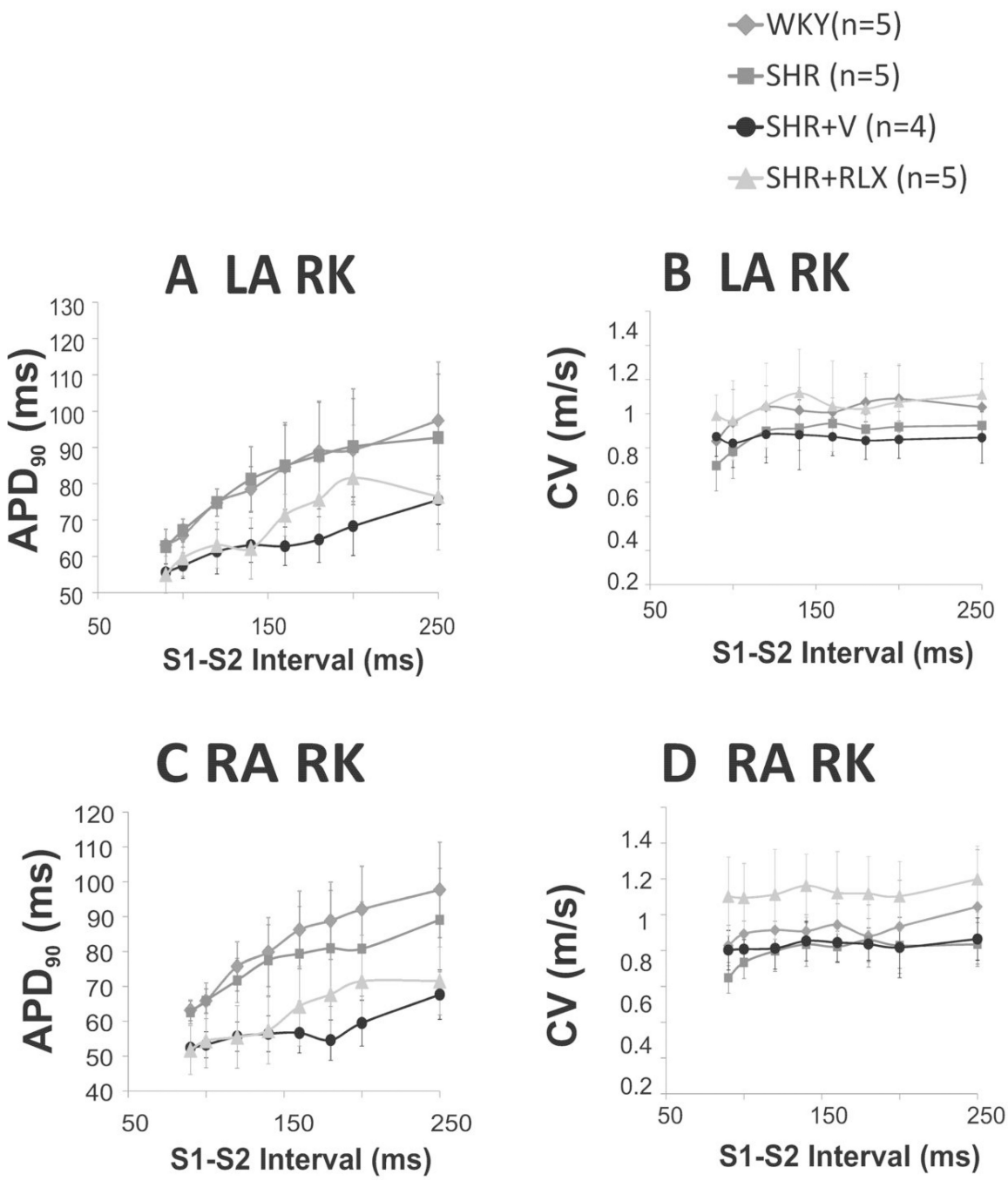

Figure 4. Restitution kinetics of Action Potential Duration (APD) and Conduction Velocity (CV)

A and **B**: Restitution kinetics (RK) measured from the left atrium (LA) for Conduction Velocity (CV) and action potential durations at 90% recovery to baseline (APD_{90}), respectively. CV and APD_{90} were measured as a function of S1–S2 interval. For APD_{90}: WKY vs. SHR, p=NS; SHR vs. SHR+V, p<0.01; and SHR vs. SHR+RLX, p<0.01. For CV: WKY vs. SHR, p< 0.05; SHR vs. SHR+V, p=NS; SHR vs. SHR+RLX, p< 0.01. **C** and **D**: Restitution kinetics from the right atrium (RA) for CV and APD_{90} respectively. CV and APD were measured as a function of S1–S2 interval. APD_{90}: WKY vs. SHR, p< 0.0; SHR

vs. SHR+V, $p<0.01$; SHR vs. SHR+RLX, $p<0.01$. CV: WKY vs. SHR, $p< 0.01$; SHR vs. SHR+V, p=NS; SHR vs. SHR+RLX, $p< 0.01$. All values are reported as mean + SD.

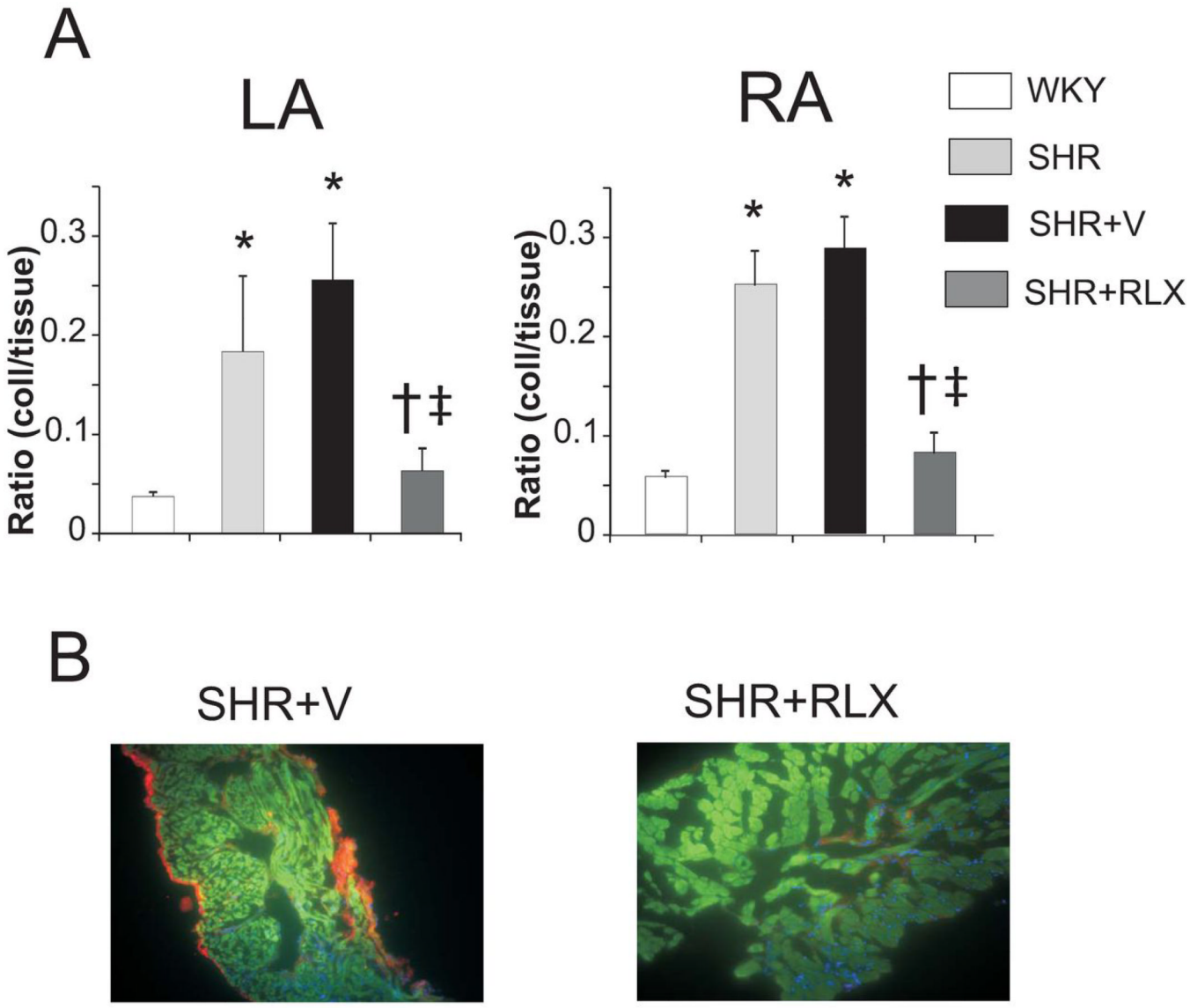

Figure 5. Fibrotic remodeling of atria and its reversal with RLX

A: LA and RA collagen I expression relative to tissue area for WKY, SHR, SHR+V, and SHR+RLX. All values are reported as mean + SD. Sample size n=3–5 per group. * $p < 0.05$ versus WKY; † $p < 0.05$ versus SHR; ‡ $p < 0.05$ versus SHR+V. **B**: Representative immuno-histological sections at 20× magnification of age-matched male LA of SHR+RLX and SHR +V. Phalloidin is represented in green; Collagen I is shown in red; nuclei (Hoechst) in blue.

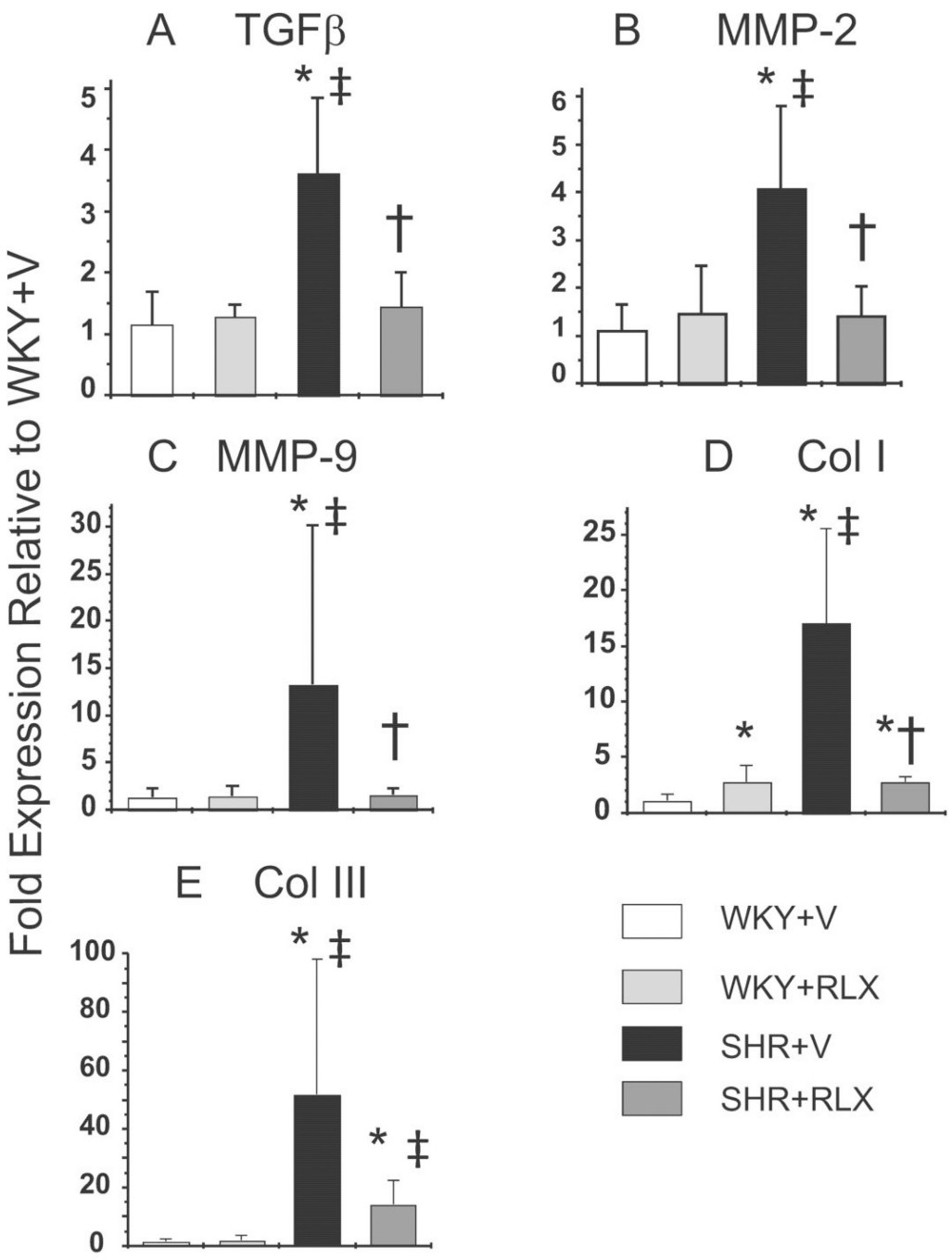

Figure 6. Relaxin treatment decreases expression of fibrosis-related transcripts

Fold expression of TGFβ, MMP-2, MMP-9, collagen I, and collagen III relative to that of WKY treated with vehicle (V) in RNA isolated from left atria (LA). RLX: relaxin treated. Values are mean ± (SD). Sample size n=4–5 per group. * $p< 0.05$ versus WKY+V; † $p< 0.05$ versus SHR+V; ‡ $p< 0.05$ versus WKY+RLX.

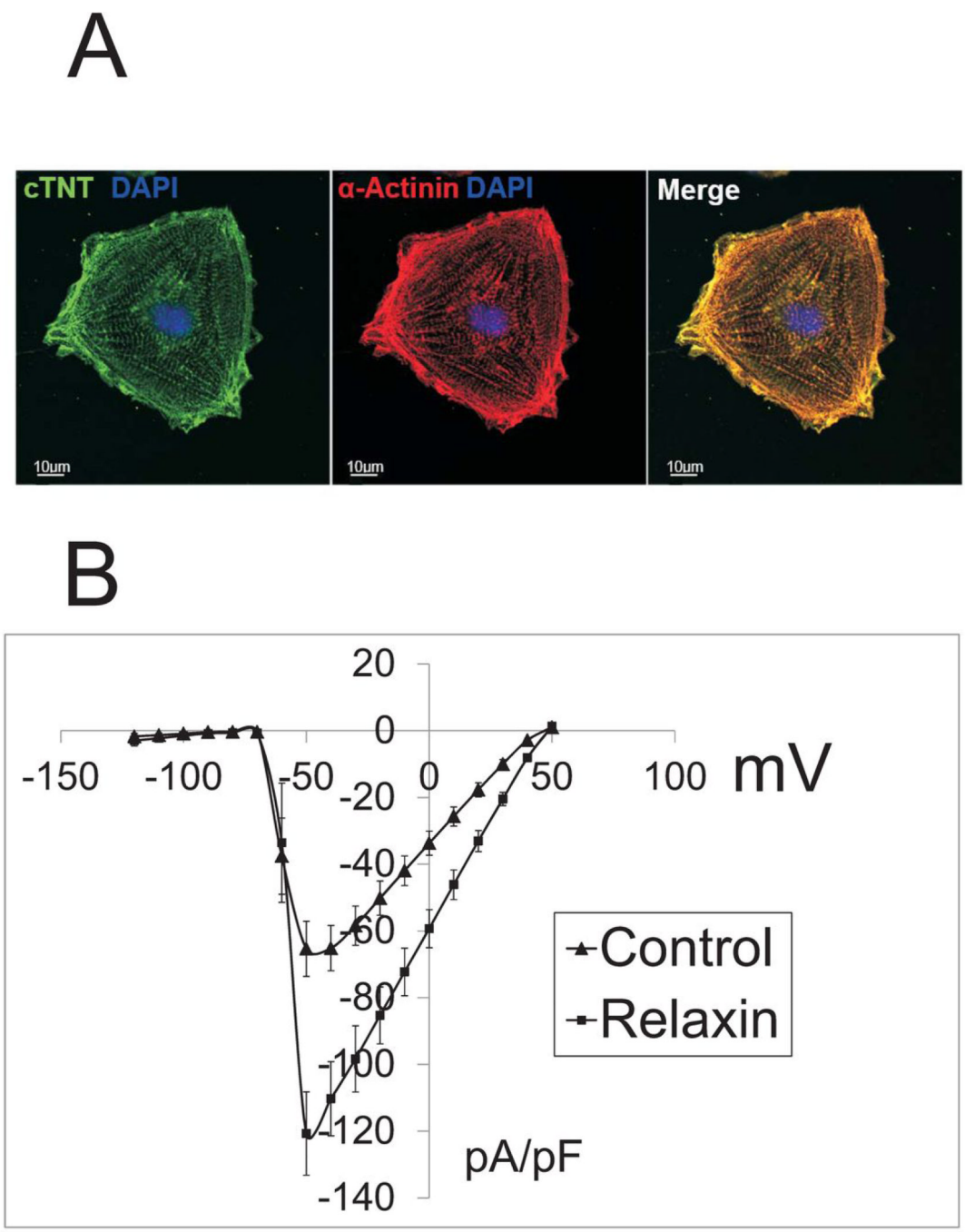

Figure 7. Relaxin treatment of human iPS-CMs doubles I_{Na} Density

Cardiomyocytes differentiated from human inducible pluripotent stem cells (iPSC) were cultured for 48 hours with a vehicle or 0.1 μM RLX. **A**: A representative image of human Y1 iPS cell derived CMs immuno-stained with anti-cTNT (Thermal Fisher) and anti-a-actinin (abcam) antibodies and counterstained with DAPI. **B**: Current-to-voltage (I-V) relationships were measured and normalized with respect to cell capacitance. I-V plots for control and RLX treated human iPS-CMs demonstrate a marked ~2-fold upregulation of Na^+ current density (n=18 for each group, p=0.0023).

Table 1

In Left Atria, effect of RLX on APD_{90}, CV and AP Rise-time vs. cycle length (CL)

CL	APD_{90}				CV				Rise Time			
	WKY n=5 ‡	SHR (n=5)	SHR+V (n=4) *	SHR+RLX (n=5) * ‡	WKY *‡	SHR	SHR+V	SHR+RLX * ‡	WKY	SHR	SHR+V	SHR+RLX
250	97±16	93±18	75±6	76±15	1.03±0.2	0.93±.03	0.86±0.2	1.17±0.1	30.5±0.4	29.8±1.3	29.1±0.1	29.4±0.4
200	89±14	90±16	68±8	82±15	1.09±0.2	0.92±0.1	0.85±0.1	1.15±.04	30.6±0.3	30.1±1.5	29.2±0.1	29.1±0.2
180	89±14	88±15	65±6	76±10	1.07±0.2	0.91±0.1	0.84±0.1	1.09±0.1	30.4±0.1	30.3±1.6	29.1±0.1	29.2±0.1
160	85±12	85±12	63±5	71±6	1.01±0.1	0.94±0.1	0.86±0.1	1.15±0.1	30.8±0.4	30.3±1.7	29.0±0.3	29.2±0.2
140	78±6	81±9	63±5	62±8	1.02±0.1	0.92±0.1	0.88±0.2	1.20±0.1	30.7±0.1	30.3±1.8	29.2±0.1	29.2±0.2
120	75±1	75±4	61+6	63±6	1.04±0.1	0.90±0.1	0.88±0.2	1.15±0.1	30.6±0.03	30.0±1.5	29.2±0.1	29.2±0.1
100	66±3	67±3	57±4	60±5	0.95±0.2	0.78±0.2	0.83±0.1	1.02±0.2	30.2±0.2	30.0±2.3	29.4±0.1	29.3±0.4
90	63±1	63±5	56±2	55±5	0.84±0.1	0.70±0.2	0.87±0.1	1.02±0.1	29±0.3	29.8±2.4	29.2±0.1	29.0±0.1

In each left atria, AP rise-time and APD_{90} was measured from 10 pixels and averaged for 5 atria; CL, AP rise-time and APD_{90} are in ms, CV in m/s, as means± SD. For WKY n= 5 SHR and SHR+RLX n= 5 hearts and for SHR + V, n=4 hearts.

*p<0.05 vs. SHR;

‡*p*< 0.05 vs. WKY, SHR and SHR+V, (ANCOVA).