Improved Chemical Synthesis and Demonstration of the Relaxin Receptor Binding Affinity and Biological Activity of Mouse Relaxin†

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ABSTRACT: The primary stored and circulating form of relaxin in humans, human gene-2 (H2) relaxin, has potent antifibrotic properties with rapidly occurring efficacy. However, when administered to experimental models of fibrosis, H2 relaxin can only be applied over short-term (2−4 week) periods, due to rodents mounting an antibody response to the exogenous human relaxin, resulting in delayed clearance and, hence, increased and variable circulating levels. To overcome this problem, the current study investigated the therapeutic potential of mouse relaxin over long-term exposure in vivo. Mouse relaxin is unique among the known relaxins in that it possesses an extra residue within the C-terminal region of its A-chain. To enable a detailed assessment of its receptor interaction and biological properties, it was chemically synthesized in good overall yield by the separate preparation of each of its A- and B-chains followed by regioselective formation of each of the intramolecular and two intermolecular disulfide bonds. Murine relaxin was shown to bind with high affinity to the human, mouse, and rat RXFP1 (primary relaxin) receptor but with a slightly lower affinity to that of H2 relaxin. When administered to relaxin-deficient mice (which undergo an age-dependent progression of organ fibrosis) over a 4 month treatment period, mouse relaxin was able to significantly inhibit the progression of collagen accumulation in several organs including the lung, kidney, testis, and skin (all $p < 0.05$ vs untreated group), consistent with the actions of H2 relaxin. These combined data demonstrate that mouse relaxin can effectively inhibit collagen deposition and accumulation (fibrosis) over long-term treatment periods.

Relaxin was first identified in 1926 as a substance influencing the reproductive tract and was subsequently found to be a peptide hormone with a two-chain structure similar to that of insulin (*1*, *2*). Relaxin is primarily produced by the corpus luteum of the ovary and/or placenta of pregnancy in most mammals where it exerts multiple essential actions on the female reproductive tract to support pregnancy, facilitate delivery, and prepare the mammary glands for lactation (*3*, *4*). More recently, our group and others have shown that locally produced relaxin has much wider actions, including roles in the cardiovascular (*5*, *6*) and central nervous systems (*7*, *8*) and an essential role in collagen turnover (*9*). These actions of relaxin are mediated via a leucine-rich repeat-containing G-protein coupled receptor, known as LGR7[1] (*10*), which has more recently been renamed relaxin family peptide receptor 1 (RXFP1) (*11*).

Humans and higher primates have three relaxin genes, termed *RLN1* (*12*), *RLN2* (*13*), and *RLN3* (*14*), while rodents only have two relaxin genes, termed *RLN1* (*15*) (rodent equivalent of human *RLN2*) and *RLN3* (*14*) (rodent equivalent of human *RLN3*). The products of the human *RLN2* and mouse *RLN1* genes, termed human gene-2 (H2) relaxin and mouse relaxin, respectively, are the major stored and circulating forms of relaxin in their respective species (*3*, *4*, *11*). Several studies have demonstrated that H2 relaxin is a potent antifibrotic agent with rapidly occurring efficacy when administered to experimental models of fibrosis in vivo (*3*−*5*, *9*). This is consistent with the fact that H2 relaxin can bind with high affinity to the mouse and rat RXFP1 receptor (*16*). However, in all studies to date, the antifibrotic actions of H2 relaxin have only been evaluated over short-term (2−4 week) treatment periods due mainly to the fact that rodents mount an antibody response to the exogenous human relaxin which results in delayed clearance and, hence, increased and variable circulating levels (*17*). When these levels are too high, the effects of H2 relaxin can be detrimental rather than beneficial (*18*).

To overcome this problem, our group has focused on synthetically producing mouse relaxin as a means of evaluating the longer term effects of exogenous relaxin in murine models of fibrosis without the interference of antibody responses. Mouse relaxin is unusual among the known

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[1] Abbreviations: cAMP, cyclic adenosine monophosphate; DMF, *N*,*N*′-dimethylformamide; DPDS, 2,2′-dipyridyl disulfide; H2, human gene-2; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HCl, hydrochloric acid; HEK, human embryonic kidney; LGR7, leucine-rich repeat-containing G-protein coupled receptor 7; LV, left ventricle; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; RP-HPLC, reversed-phase high-performance liquid chromatography; RV, right ventricle; RXFP1, relaxin family peptide receptor 1; TES, triethylsilane; TFA, trifluoroacetic acid; Tyr, tyrosine; TFMSA, trifluoromethanesulfonic acid.

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relaxins in that it possesses an extra amino acid residue, tyrosine (Tyr), within the C-terminal region of its A-chain. Using solid-phase peptide synthesis, Büllesbach and Schwabe prepared both native mouse relaxin and an analogue lacking this residue (*19*) and observed that while both peptides were active in the pubic symphysis and brain relaxin receptor binding assays, the analogue lacking Tyr was significantly more active than the native peptide. We recently established improved regioselective disulfide bond formation methods for the efficient preparation of insulin-like peptides (*16*, *20*, *21*) that involve fewer intermediate steps and have also constructed cell lines that express mouse, human, and rat RXFP1 receptors (*16*) to more comprehensively assess the efficacy of these peptides.

In this study, we used these improved methods to chemically synthesize mouse relaxin and systematically evaluate (i) its receptor binding activity using mouse, rat, and human LGR7 expressing cell lines. Furthermore, (ii) the antifibrotic effects of the synthetic mouse relaxin were determined in vivo when administered over a long-term (4 month) treatment period to a murine model of progressive fibrosis (*22*). These studies were completed so as to more precisely determine the specificity of mouse relaxin in relation to other relaxin peptides.

EXPERIMENTAL PROCEDURES

Solid-Phase Peptide Synthesis. The selectively S-protected A- and B-chains (of mouse relaxin) were assembled as their C-terminal acids by the continuous flow Fmoc solid-phase synthesis methodology, essentially as previously described (*16*). The solid support was Fmoc-Cys(Acm)-NovaSyn TGT (Novabiochem) for the A-chain, and Fmoc-Leu-PAC-PEG-PS-terminal amino acid-linked PAC-PEG-PS (PerSeptive Biosystems) and HBTU-activated Fmoc-amino acids were used throughout. Amino acid side chain protection was afforded by the following: Arg, Pbf; Gln, Trt; Asp and Glu, O-Bu^t; Tyr, Bu^t; His, Trt; Lys, Boc; Ser, Bu^t; and Trp, Boc. For the A-chain peptide, S-protection was afforded by Trt (Cys^{10,15}), Acm (Cys^{25}), and Bu^t (Cys^{11}). The N-terminal residue of the A-chain was coupled as pyroglutamic acid. For the B-chain, Trt (Cys^{14}) and Acm (Cys^{26}) were used. All amino acid derivatives were purchased from Auspep (Melbourne, Australia). N^{α}-Fmoc deprotection was with 20% piperidine in DMF. Assembly of both the A- and B-chain peptides commenced on 0.125 and 0.10 mmol scales, respectively, using a 4-fold excess of activated amino acid and 30 min coupling times. No repeat couplings were carried out. After acylation and deprotection of the final residues, cleavage from the solid supports and side chain deprotection were achieved by a 2.5 h treatment of the two separate peptide resins with trifluoroacetic acid (TFA) and, for the B-chain, in the presence of phenol, thioanisole, ethanedithiol, and water (82.5/5/5/2.5/5, v/v) with a few drops triethylsilane (TES). For the A-chain, cleavage was with TFA in the presence of ethanedithiol, water, and TES (95/2/2/1). The resulting crude peptides were subjected to preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C18 column (Hesperia) using a 1%/min gradient of CH_3CN in 0.1% aqueous TFA.

A-Chain Intramolecular Disulfide Oxidation. Crude cleaved [Cys^{10,15}(*S*-thiol), Cys^{11}(But), Cys^{25}(Acm)]-A-chain (976 mg, 335 μmol) was dissolved in 0.1 M Gly−NaOH, pH 8.5 (2.5 L), and to this was added 1 mM 2-dipyridyl disulfide (DPDS) in MeOH (214 mL, 214 μmol) (*23*). Oxidation was completed after 2 h as monitored by analytical RP-HPLC. The solution was acidified by addition of neat TFA, and then the peptide was isolated by preparative RP-HPLC and subsequent freeze-drying to give 168.2 mg (59.2 μmol, 27%) of purified [Cys^{11}(But), Cys^{25}(Acm)]-A-chain.

[Cys^{11}(Pyr), Cys^{25}(Acm)]-A-Chain. Intramolecular disulfide-bonded [Cys^{11}(But), Cys^{25}(Acm)]-A-chain (168 mg, 59.1 μmol) was converted to the Cys^{11} *S*-pyridinylsulfenyl form by treatment with DPDS (*23*) in neat TFA (5.0 mL) containing thioanisole (0.5 mL), chilled to ≤0 °C, before 5.0 mL of TFMSA/TFA (1/5 v/v) was added and stirred for 30 min, maintaining the temperature at or below 0 °C. The peptide was then precipitated in ether and the pellet suspended in 6 M GdHCl, pH 8.0, for purification. The target peptide was isolated by preparative RP-HPLC to give 148.6 mg (86.8%).

Combination of [Cys^{11}(Pyr), Cys^{25}(Acm)]-A-Chain with [Cys^{14}(S-Thiol), Cys^{26}(Acm)]-B-Chain. A-chain peptide (120.9 mg, 41.8 μmol) was dissolved in 0.1 mM NH_4HCO_3 or 8 M GdHCl, pH 8.5 (7.0 mL), and added to crude B-chain (153.0 mg, 37.4 μmol) in the same buffer (11 mL). The mixture was stirred vigorously at 37 °C and the reaction monitored by analytical RP-HPLC. After 30 min (or 24 h if using the GdHCl buffer), the reaction was terminated by addition of glacial acetic acid and the target product isolated by preparative RP-HPLC to give 57.6 mg (8.4 μmol, 10.5%).

Purification of Mouse Relaxin. The [Cys^{25}(Acm)]-A-chain/[Cys^{26}(Acm)]-B-chain (57.6 mg, 8.4 μmol) was dissolved in glacial acetic acid (26.6 mL) and 80 mM HCl, and to this was added dropwise 25.2 mL of 20 mM iodine/acetic acid (0.50 mmol). After 1 h, the reaction was stopped by addition of 25.2 mL of 20 mM ascorbic acid. Preparative RP-HPLC, as described above, was then used to isolate and purify the product (23.7 mg, 3.50 μmol, 42.0% yield, 4.4% overall relative to starting crude B-chain).

Characterization of Mouse Relaxin. The purity of the synthetic mouse relaxin peptide was assessed by analytical RP-HPLC and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry using a Bruker Autoflex II instrument (Bremen, Germany) in the linear mode at 19.5 kV. Peptide quantitation was determined by amino acid analysis of a 24 h acid hydrolyzate using a GBC instrument (Melbourne, Australia).

Binding Assays. HEK-293T cells expressing human (*24*), rat, and mouse LGR7 (*25*) were tested for their ability to bind mouse relaxin in comparison to H2 relaxin (recombinant peptide provided by BAS Medical, San Mateo, CA). Whole cell binding assays in 24-well plates using ^{33}P-labeled H2 relaxin were performed as previously described (*26*). All data were analyzed using GraphPad PRISM (GraphPad Software, San Diego, CA). A nonlinear regression one-site binding model was used to obtain pIC_{50}. All values were expressed as the mean ± standard error from three separate observations.

Animals. Male relaxin wild-type (*Rln*+/+) and relaxin gene-knockout (*Rln*−/−) litter mate mice used in this study were generated, individually genotyped, and housed in a controlled environment as described before (*27*). Two-month-old *Rln*+/+ and *Rln*−/− mice, with equivalent baseline

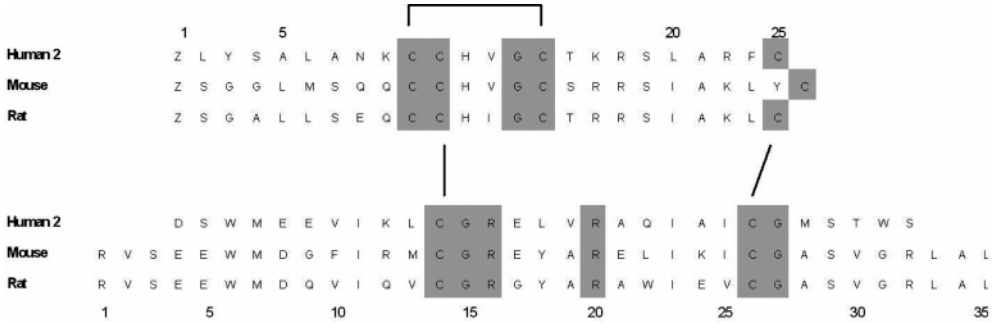

FIGURE 1: Amino acid composition of the mouse relaxin structure and comparative alignment to the human relaxin-2 and rat relaxin A- and B-chains.

collagen levels in the lung (*17*), heart (*28*), kidney (*29*), and skin (*30*), were used for subsequent experimentation and analysis. Untreated *Rln*+/+ ($n = 7$) and *Rln*−/− mice ($n = 7$) were aged until 6 months, when *Rln*−/− animals were previously shown to demonstrate pulmonary (*17*), renal (*29*), dermal (*30*), and testicular (*31*) fibrosis. Separate groups of 2-month-old *Rln*−/− mice were also treated with a high dose ($n = 6$) or low dose ($n = 5$) of mouse relaxin over a 4 month period. These experiments were approved by the Howard Florey Institute's Animal Ethics Committee, which adheres to the Australian Code of Practice for the care and use of laboratory animals for scientific purposes.

Osmotic Minipump Implantation in Mice. Two-month-old *Rln*−/− mice undergoing mouse relaxin treatment were anesthetized with Isoflurane (Halocarbon Products Corp., River Edge, NJ), and a small full-thickness cut was made on their dorsal surface. A subcutaneous pouch was made by blunt dissection before miniosmotic pumps (model 2004; Alzet, Cupertino, CA) were inserted into each mouse. These pumps were used to continuously infuse the mouse relaxin into the circulation (based on its short half-life of 10−15 min) up to 31 days in length. Subgroups of *Rln*−/− mice were administered with either a high dose (100 μg kg^{-1} day^{-1}; $n = 6$) or 10-fold lower dose (10 μg kg^{-1} day^{-1}; $n = 5$) of mouse relaxin. At the end of each month (30 or 31 day period), the empty osmotic minipumps were carefully removed from each *Rln*−/− mouse, before a new set of relaxin-filled pumps were inserted into the same subcutaneous pouch of each respective animal, under anesthesia. This procedure was repeated monthly, until all relaxin-treated mice were aged to 6 months (i.e., at which point they would have received the mouse relaxin over a 4 month period). Untreated *Rln*−/− and *Rln*+/+ animals were also aged until 6 months, acting as appropriate controls.

Tissue Collection. All mice were weighed and killed at 6 months of age for tissue collection. Serum was immediately isolated from each mouse by cardiac puncture before the lung, heart, kidneys, testes, and skin were rapidly excised, washed in ice-cold PBS, blotted, and weighed. The heart was dissected into the left ventricle (LV), right ventricle (RV), and atria, while the kidney was further separated into the cortex and medulla. Tissues were either snap-frozen in liquid nitrogen and stored at −80 °C for hydroxyproline analysis or fixed in 4% paraformaldehyde for histological analysis.

Hydroxyproline Analysis of Collagen. The collected organs from each mouse were lyophilized to dry weight and treated as described before (*32*) to determine their hydroxyproline contents. Hydroxyproline values were converted to collagen content by multiplying by 6.94 (based on hydroxyproline representing 14.4% of the amino acid composition of collagen in most mammalian species) and further expressed as a proportion of the dry weight tissue (collagen concentration), respectively.

Histology. For histological observations, paraformaldehyde-fixed tissues (lung, kidney cortex, skin) were paraffin embedded, cut into 5 μm sections, and stained with 0.1% Picrosirius red (which specifically stains collagen red). Representative images of each organ from untreated *Rln*+/+ and *Rln*−/− mice and 100 μg kg^{-1} day^{-1} mouse relaxin-treated *Rln*−/− mice were captured with an CCD video camera (Optimas Bioscan, Edmonds, WA) and digitized using the Image-Pro Plus 6.0 program (Media Cybernetics, Silver Spring, MD). Morphometric analysis was also performed on skin sections from the three groups of mice ($n = 5$ mice per group), to quantify the level of collagen staining. Skin sections were sampled in a systematic fashion, and 6−8 fields from each sample were analyzed using the Image-Pro Plus 6.0 program. The Picrosirius red-stained area was calculated as a percentage of the total area within each field, and the mean ± SE percentage obtained.

Statistical Analysis. The results were analyzed by one-way ANOVA, using the Newman−Keuls test for multiple comparisons between groups. All data in this paper are presented as the mean ± SEM, with $p < 0.05$ described as statistically significant.

RESULTS

Synthesis and Characterization of Mouse Relaxin. The primary structure of mouse relaxin (Figure 1) comprises a 25-residue A-chain linked via insulin-like disulfide bond pairings to a 35-residue B-chain. Like other relaxins and members of the insulin superfamily, it contains three disulfide bonds in the characteristic insulin pairing pattern. However, it differs from other relaxins in its overall amino acid composition and in that its A-chain contains an extra residue adjacent to the C-terminal amino acid.

The mouse relaxin was generated by highly efficient regioselective disulfide bond solid-phase peptide synthesis of the A- and B-chains in which pairs of cysteines were selectively *S*-thiol-protected with masking groups that allowed their individual removal and subsequent stepwise disulfide bond formation. The intramolecular disulfide bond within the A-chain was formed by oxidation of the free thiol groups of the Cys^{10,15} residues with DPDS. The Cys^{11} side chain protecting group was then converted to the *S*-pyridinyl

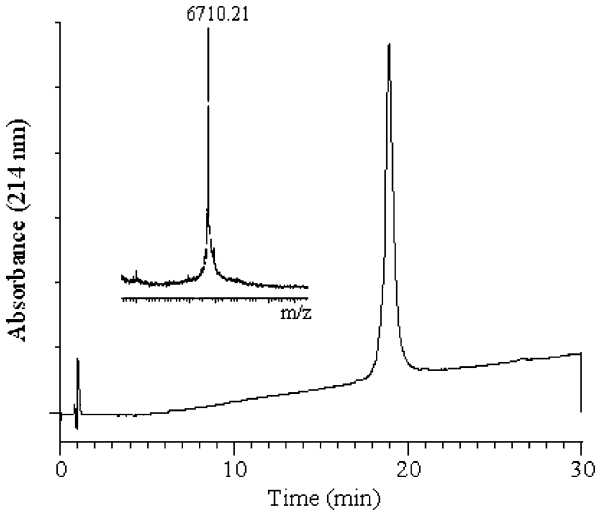

FIGURE 2: Analytical RP-HPLC of synthetic mouse relaxin. Conditions: column, Waters C18 (3.9 × 150 mm); buffer A, 0.1% aqueous TFA; buffer B, 0.1% TFA in CH_3CN; gradient, 20−50% buffer B in 30 min; flow rate, 1.5 mL/min. Inset: MALDI-TOF mass spectrometry of synthetic mouse relaxin was used to confirm a purified product with a molecular mass (*m*/*z*) of 6710.21 Da, which was consistent with the theoretical value of 6710.00 Da.

Table 1: Receptor Binding Affinities of Mouse, Rat, and Human (H2) Relaxins

receptor	peptide	pIC_{50}[a]	*n*
mouse RXFP1	H2 relaxin[b]	9.71 ± 0.02	3
	mouse relaxin	9.14 ± 0.05	3
	rat relaxin[b]	8.89 ± 0.06	3
human RXFP1	H2 relaxin[b]	10.04 ± 0.37	3
	mouse relaxin	9.11 ± 0.05	3
	rat relaxin[b]	7.98 ± 0.34	3
rat RXFP1	H2 relaxin[b]	9.46 ± 0.06	3
	mouse relaxin	8.96 ± 0.04	3
	rat relaxin[b]	9.06 ± 0.19	3

[a] Data are the means ± SEM of three separate experiments. [b]Values obtained from refs *25* and *37*.

group by reaction of the peptide with DPDS in the presence of trifluoromethanesulfonic acid, after which the resulting product was combined with the B-chain. The free thiol group of the Cys^{14} led to thiolysis of the *S*-pyridinyl group of the A-chain forming the first intermolecular disulfide bond. The resulting di-Acm-containing peptide was treated with iodine in hydrochloric acid to generate the second, and final, intermolecular disulfide bond. The overall yield of the mouse relaxin following HPLC purification was about 4% relative to the starting B-chain peptide, which is satisfactory given the complexity of the peptide and the handling difficulties associated with the poor solubility of the starting B-chain. It was confirmed to be of high purity by MALDI-TOF MS (calcd 6710.00Da; found 6710.21Da) and analytical RP-HPLC (Figure 2). Tryptic mapping followed MALDI-TOF MS analysis of the resulting fragments confirmed the parallel alignment of the two chains (data not shown).

Affinity of Mouse Relaxin to Its Primary Receptor, RXFP1. The ability of the synthetic mouse relaxin to bind to the respective mouse, rat, and human RXFP1 receptors was tested for the first time and compared to the ability of H2 relaxin and rat relaxin to bind to these receptors (Table 1). Results of mouse relaxin vs H2 relaxin binding to mouse and human RXFP1 (Figure 3) and the pooled pIC_{50} results

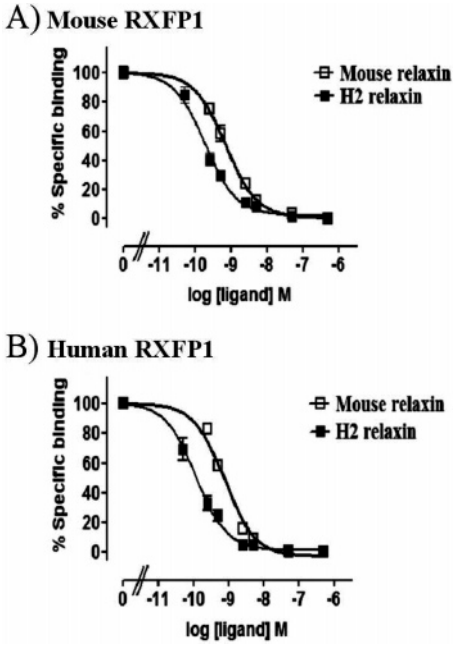

FIGURE 3: cAMP accumulation in response to relaxin in stably transfected (A) mouse LGR7 and (B) human LGR7 cells. Activities are plotted as the percent H2 relaxin maximum responses, respectively. Stimulation of LGR7 results in increased cellular cAMP.

Table 2: Determination of the Extent of Fibrosis in the *Rln*−/− Mouse: A Model of Progressive Fibrosis[a]

	collagen concn[b]				
	previous studies				current study
organ	6 months	9 months	12 months	ref	6 months
lung	19	23	30	*17*	19
LV (heart)		19	17	*28*	14[c]
kidney cortex	19		21	*29*	18
testis	19			*31*	18
skin	40		56	*30*	44

[a] All values are expressed as the percentage increase in collagen concentration (fibrosis) in each organ of *Rln*−/− mice, compared to respective measurements obtained from age-matched *Rln*+/+ animals, at each time point indicated. [b] % collagen content/dry weight tissue. [c] Percentage of collagen concentration was not significantly different from that measured in age-matched *Rln*+/+ mice, while all other values were significantly ($p < 0.05$) different from measurements obtained from age-matched *Rln*+/+ animals.

(Table 1) are shown. The rank order of potency of mouse relaxin at both human and mouse RXFP1 was H2 > mouse > rat relaxin. In contrast, rat relaxin had a higher affinity for rat RXFP1 than the mouse peptide but had less affinity than the H2 relaxin for the same receptor (Table 1).

Determination of the Antifibrotic Effects of Mouse Relaxin. Six-month-old male Rln−/− mice developed increased collagen concentration (fibrosis) of the lung (by 19%), kidney cortex (by 18%), testis (by 18%), and skin (by 44%) (all $p < 0.05$) compared to that measured in age-matched Rln+/+ mice, consistent with previous reports (Table 2, Figure 4). At this time point though, only a trend toward increased left ventricular (cardiac) fibrosis was observed (by 14%, $p > 0.05$), as determined by hydroxyproline analysis. The administration of 10 μg kg^{-1} day^{-1} synthetic mouse relaxin to Rln−/− mice over a 4 month period had no significant effects on the progression of fibrosis in any of the organs studied (Figure 4). However, when administered at a 10-

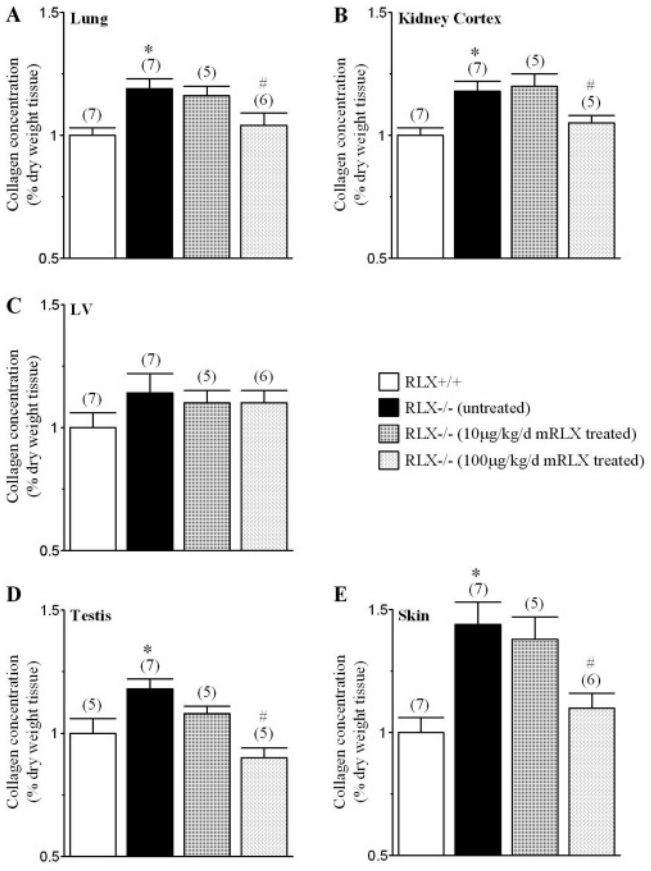

FIGURE 4: Total collagen concentration (percent collagen content/dry weight tissue) in the lung (A), kidney cortex (B), left ventricular myocardium (C), testis (D), and skin (E) of 6-month-old male untreated *Rln*+/+ mice, untreated *Rln*−/− mice, and *Rln*−/− mice treated with either 10 or 100 μg kg^{-1} day^{-1} mouse relaxin over a 4 month period (from 2 to 6 months of age). Numbers in parentheses represent the number of animals analyzed per group. $^{*}p < 0.05$ vs values from age-matched *Rln*+/+ mice; $^{\#}p < 0.05$ vs values from untreated *Rln*−/− mice.

fold higher concentration (100 μg kg^{-1} day^{-1}) over the same time period, the exogenous mouse relaxin was able to significantly inhibit the progression of fibrosis in the lung (Figure 4A), kidney cortex (Figure 4B), testis (Figure 4D), and skin (Figure 4E) to levels measured in Rln+/+ animals (all $p < 0.05$ vs respective values in untreated Rln−/− animals).

Picrosirius red staining of various organs of untreated *Rln*−/− mice demonstrated increased collagen staining (fibrosis) surrounding the bronchioles and arterioles of the lung and within the interstitial tubules and glomeruli of the kidney cortex (Figure 5A). Collagen staining was also increased to a greater extent in the dermis of untreated *Rln*−/− mice (Figure 5), which superseded the hypodermal layer of these animals, compared to that observed in untreated *Rln*+/+ animals. Consistent with the hydroxyproline analysis data (Figure 4), the administration of 100 μg kg^{-1} day^{-1} mouse relaxin to *Rln*−/− mice, over a 4 month treatment period, resulted in a significant decrease in collagen staining within lung, kidney, and skin (Figure 5) to levels found in *Rln*+/+ animals. Interestingly, in mouse relaxin-treated skin sections, a reduced level of collagen content (Figure 5B) was associated with decreased collagen fiber density and orientation (Figure 5A).

DISCUSSION

This is the first study to demonstrate the antifibrotic potential of mouse relaxin in a murine model of progressive fibrosis, following the development of recently improved methods (*16*, *20*, *21*) by our group to successfully chemically synthesize it. The chemical preparation of a two-chain, three-disulfide-bonded polypeptide remains a significant undertaking and one which is further compounded by the intractable nature of the mouse relaxin B-chain which is poorly soluble in most aqueous solutions. It is for this latter reason that previous attempts in our laboratory to produce murine relaxin by random combination of the individual S-reduced A- and B-chains did not meet with success. The development of efficient regioselective disulfide synthesis protocols has greatly enhanced our capacity to study the biology of relaxin family peptides and was employed to successfully produce mouse relaxin in quantities that allowed for its detailed biological study. Overall yields continued to be low but satisfactory given the complexity and size of the relaxin molecule. Comprehensive chemical characterization of synthetic mouse relaxin including by analytical RP-HPLC and MALDITOF mass spectroscopy confirmed its high integrity. Tryptic mapping followed by MS analysis further confirmed the disposition of the disulfide bonds (results not shown).

The synthetic mouse relaxin was tested for its ability to bind to the mouse, rat, and human RXFP1 receptors and demonstrated a high affinity for all of these receptors albeit with slightly lower affinity than that of H2 relaxin. These data are consistent with a previous study where it was demonstrated that mouse relaxin had a slightly lower affinity for mouse brain relaxin receptors than porcine relaxin (*19*). These results clearly demonstrate that mouse relaxin will bind to the mouse RXFP1 receptor with nanomolar affinity consistent with it being the endogenous ligand for RXFP1 in mice.

Having successfully synthesized mouse relaxin and characterized its ability to bind to its primary receptor, RXFP1, we were then able to determine its antifibrotic potential over a long-term treatment period. Previously, only the antifibrotic effects of H2 relaxin (reviewed in refs *3*, *4*, and *9*) and, to a lesser extent, H3 relaxin (*33*) had been evaluated in preclinical and clinical models of disease. Both of these forms of human relaxin were shown to bind with high affinity to the rodent RXFP1 receptors (*16*) in addition to having potent and rapidly occurring effects when administered to several experimental models of fibrotic disease (reviewed in refs *3*, *4*, and *9*). Furthermore, the human relaxins were preferentially used over mouse relaxin as a multitude of ovaries from pregnant mice would need to have been isolated to generate sufficient amounts of the natural peptide for experimental studies. However, the limiting factor in all studies involving either H2 or H3 relaxin was the fact that the rodent models tested would always generate nonneutralizing antibodies to the human antigens, resulting in increased and variable circulating levels of relaxin. These increased levels of circulating relaxin were shown to be detrimental rather than beneficial in some cases (*18*). Thus, the administration of human relaxin to rodent models only allowed for short-term (10−28 day) treatment protocols. The availability of synthetic mouse relaxin has allowed us to overcome each of these problematic factors.

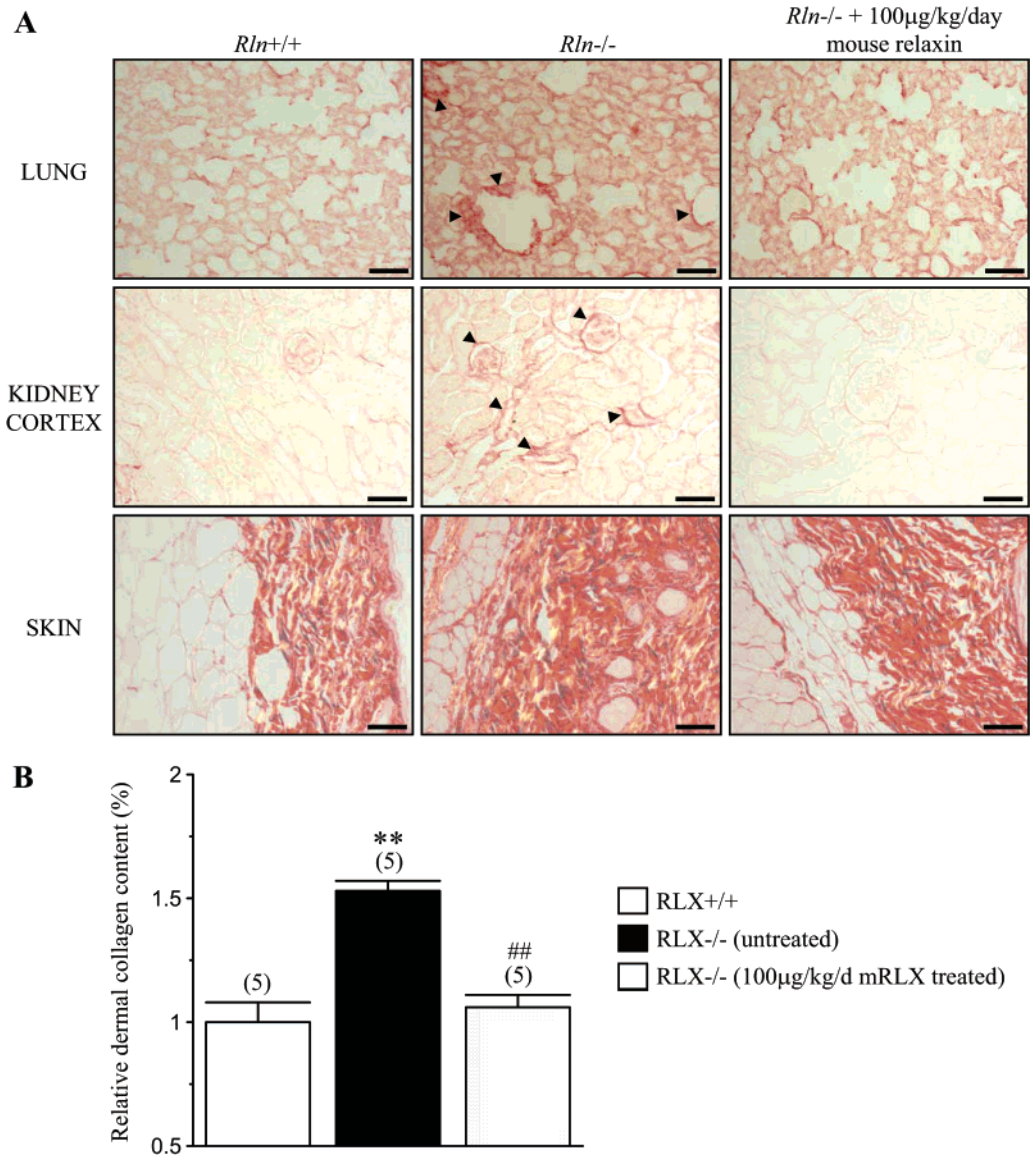

FIGURE 5: Representative Picrosirius red-stained sections (A) from the lung, kidney cortex, and skin of 6-month-old male untreated *Rln*+/+ mice, untreated *Rln*−/− mice, and *Rln*−/− mice treated with 100 μg kg^{-1} day^{-1} mouse relaxin over a 4 month period. Bar = 100 μm. The relative mean ± SE of dermal collagen content (percent total area) (B) as determined by morphometric analysis of skin sections from *Rln*+/+, *Rln*−/−, and 100 μg kg^{-1} day^{-1} treated *Rln*−/− mice is also shown. Numbers in parentheses represent the number of animals analyzed per group. $^{**}p < 0.01$ vs values from age-matched *Rln*+/+ mice; $^{\#\#}p < 0.01$ vs values from untreated *Rln*−/− mice.

The findings of our study demonstrated that the long-term administration of mouse relaxin to relaxin-null mice, which undergo an age-related progression of fibrosis in most organs by 6 months of age (*17*, *28*−*31*), was able to markedly inhibit the progression of collagen accumulation in several organs including the lung, kidney, testis, and skin. This is the first study to demonstrate that relaxin treatment can be sustained for such a long-term (4 month) period, without any noticeable side effects to the animals. While the circulating levels of mouse relaxin could not be detected due to the lack of existing bioassays, our study showed that (i) the antifibrotic effects of mouse relaxin were dose-dependent and (ii) a 5-fold lower dose of mouse relaxin could be used to inhibit long-term collagen accumulation as compared to the concentrations of H2 relaxin, used over shorter term treatment periods (*17*, *18*, *26*, *29*, *30*, *34*−*36*). Given the similar affinities of mouse and H2 relaxin for the mouse RXFP1 receptor, it is likely that they have very similar activities in vivo and therefore H2 relaxin will also be as effective at this lower dose.

This study provides proof of principle that the effects of mouse relaxin are similar to those previously reported for H2 (*17*−*18*, *26*, *29*−*30*, *34*−*36*) and H3 (*16*, *33*) relaxin and suggests that several relaxin peptides have potent collagen remodeling effects, regardless of the species they were derived from. These findings have important clinical potential for the long-term use of H2 relaxin in humans, particularly involving patients with genetic defects that lead to progressive and ongoing fibrosis. The demonstration that the overexpression of a single peptide (known to regulate collagen turnover) was able to suppress the progression of fibrosis in multiple organs further confirms the therapeutic significance of relaxin (*5*, *9*). Several studies have already demonstrated that relaxin acts at multiple levels to inhibit organ fibrosis (*9*, *18*, *26*, *34*−*36*), of which its primary mode of action involves the inhibition of transforming growth

factor-β-induced collagen production in addition to the stimulation of matrix metalloproteinase-induced collagen breakdown. Further work is now required to evaluate the antifibrotic effects of mouse relaxin in other murine models of experimental fibrosis, particularly those involving more chronic phases of organ scarring.

In conclusion, we found that the administration of synthetic mouse relaxin to a murine model of progressive fibrosis was able to significantly inhibit long-term collagen accumulation. The collagen remodeling actions of mouse relaxin were shown to be consistent with that previously reported for H2 and H3 relaxin and were also shown to be associated with a high binding affinity to RXFP1, the primary relaxin receptor. Generating mouse relaxin by synthetic means has allowed us to both overcome certain limitations associated with using human relaxins and to demonstrate the therapeutic significance of the peptide.

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