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Relaxin-1–deficient mice develop an age-related progression of renal fibrosis

CHRISHAN S. SAMUEL, CHONGXIN ZHAO, COURTNEY P. BOND, TIM D. HEWITSON, EDWARD P. AMENTO, and ROGER J. SUMMERS

Howard Florey Institute of Experimental Physiology & Medicine, The University of Melbourne, Melbourne, Victoria, Australia; Department of Pharmacology, Monash University, Melbourne, Victoria, Australia; Department of Nephrology, The Royal Melbourne Hospital, Melbourne, Victoria, Australia; Molecular Medicine Research Institute, Sunnyvale, California; and Stanford University School of Medicine, Stanford, California

Relaxin-1–deficient mice develop an age-related progression of renal fibrosis.

Background. Relaxin (RLX) is a peptide hormone that stimulates the breakdown of collagen in preparation for parturition and when administered to various models of induced fibrosis. However, its significance in the aging kidney is yet to be established. In this study, we compared structural and functional changes in the kidney of aging relaxin-1 (RLX−/−) deficient mice and normal (RLX+/+) mice.

Methods. The kidney cortex and medulla of male and female RLX+/+ and RLX−/− mice at various ages were analyzed for collagen content, concentration, and types. Histologic analysis, reverse transcription-polymerase chain reaction (RT-PCR) of relaxin and relaxin receptor mRNA expression, receptor autoradiography, glomerular isolation/analysis, and serum/urine analysis were also employed. Relaxin treatment of RLX−/− mice was used to confirm the antifibrotic effects of the peptide.

Results. We demonstrate an age-related progression of renal fibrosis in male, but not female, RLX−/− mice with significantly ($P < 0.05$) increased tissue dry weight, collagen (type I) content and concentration. The increased collagen expression in the kidney was associated with increased glomerular matrix and to a lesser extent, interstitial fibrosis in RLX−/− mice, which also had significantly increased serum creatinine ($P < 0.05$) and urinary protein ($P < 0.05$). Treatment of RLX−/− mice with relaxin in established stages of renal fibrosis resulted in the reversal of collagen deposition.

Conclusion. This study supports the concept that relaxin may provide a means to regulate excessive collagen deposition during kidney development and in diseased states characterized by renal fibrosis.

Progressive renal disease is characterized by glomerulosclerosis [1] and tubulointerstitial fibrosis [2, 3], which results in the formation of scar tissue and kidney dysfunction. At the onset of renal fibrosis there is activation of extracellular matrix (ECM)–producing fibroblasts, which differentiate and acquire features of smooth muscle [4]. Differentiated fibroblasts (myofibroblasts) contribute to the process of scarring and cause hyperproliferation, increased production of several ECM proteins [5], particularly collagen and reorganization of the ECM. While a number of therapies have been extensively used in progressive renal disease [6, 7], most have been limited or ineffective in reversing the connective tissue deposition (fibrosis), while others inhibit several mechanisms and are nonspecific, emphasizing the need for novel antifibrotic therapies.

Relaxin (RLX) is a small dimeric peptide hormone with known antifibrotic properties. While relaxin is primarily produced from the pregnant ovary and prostate of mammals and has several functions that are generally associated with female reproductive tract physiology [8], its ability to inhibit excessive collagen accumulation in various cell culture and animal models of fibrosis, has been extensively studied [9–13]. Relaxin acts directly on transforming growth factor-β (TGF-β)–stimulated human dermal fibroblasts [9] and lung fibroblasts [10] to promote the decrease of types I and III collagen synthesis and deposition. In addition, relaxin has been used to decrease collagen accumulation in several rodent models of fibrosis [10, 11], including a bromoethylamine-induced model of chronic papillary necrosis [12] and two models of renal injury, caused by mass reduction [13]. These findings demonstrated that relaxin possessed antifibrotic properties in the kidney.

Mice have two relaxin genes, known as relaxin-1 [14] and relaxin-3 [15]. Relaxin-1 is the mouse equivalent of human-2 relaxin [14] and is the major stored and circulating form of relaxin in the mouse, while relaxin-3 is the mouse equivalent of the recently discovered human-3 relaxin [15]. To aid our understanding of the physiologic

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significance of relaxin-1, our group used gene targeting to establish a relaxin-1 gene knockout mouse, which lacks relaxin-1 protein [16]. Our subsequent findings demonstrated that relaxin-1–deficient mice had poor mammary gland, nipple, and female reproductive tract development during late pregnancy due to an increased collagen concentration in these tissues [16, 17]. Furthermore, relaxin-1–deficient mice underwent an age-related progression of fibrosis in the male reproductive tract [18], lung [19], and heart [20], confirming that relaxin-1 was a naturally occurring regulator of collagen turnover during development and pregnancy. However, the significance of relaxin-1 deficiency on kidney phenotypes of these mice is yet to be established.

The kidney has traditionally not been considered a source or target tissue of relaxin. However, recent studies have demonstrated relaxin mRNA expression in the mouse kidney [15], while relaxin receptor (LGR7) and relaxin-like factor/insulin-3 receptor (LGR8) gene transcripts were identified in the human kidney [21]. These findings suggest that the kidney may not only serve as a target for relaxin activity through its actions on LGR7, but may act as a source of local relaxin production in mammals. In the present study, we used relaxin-1 gene knockout mice to examine the long-term effects of relaxin deprivation on the structure and function of the kidney with the specific aim of determining whether the lack of relaxin affects renal collagen deposition. We also investigated the effects of relaxin treatment in relaxin null mice with established renal fibrosis.

METHODS

Reagents

Recombinant human relaxin (rH2) was generously provided by the Connetics Corporation (Palo Alto, CA, USA) and is bioactive in mice [22].

Animals

All male and female relaxin wild-type (RLX+/+), relaxin heterezygous (RLX+/−), and relaxin knockout (RLX−/−) mice used in this study were generated from RLX+/− (C57Blk6Jx129SV) parents [16]. The animals were housed in a controlled environment and maintained on a 14-hour light, 10-hour dark schedule with access to rodent lab chow (Barastock Stockfeeds, Pakenham, Victoria, Australia) and water. These experiments were approved by the Howard Florey Institute's Animal Experimental Ethics Committee, which adheres to the Australian Code of Practice for the care and use of laboratory animals for scientific purposes.

Tissue collection

RLX+/+ and RLX−/− male mice were obtained at 1 month, 6 months, and 12 months of age, while RLX+/+ and RLX−/− female mice were obtained at 1 month and 12 months of age. Additional female mice (including RLX+/− mice) were also obtained at 19 months of age ($N = 6$ to 8 mice per genotype and gender). All mice were weighed, before being euthanized for blood and tissue collection. Both kidneys were collected from each animal and individual tissues were immediately weighed (wet weight), before being separated into cortex and medulla. Tissues were then either stored at −80°C for hydroxyproline analysis and RNA analysis or fixed in 10% formalin for histologic analysis. For hydroxyproline analysis, separated cortex and medulla tissue were lyophilized to dry weight. An additional set of 9-month-old male RLX+/+ and RLX−/− mice ($N = 12$ to 14 per genotype) were used for other analyses (determination of collagen types, glomerular isolation and analysis, autoradiography of relaxin receptor binding, serum/urine analysis) as detailed below.

Hydroxyproline analysis of kidney tissues

The separated cortex and medulla from RLX+/+ and RLX−/− male and female mice, respectively, from the different age groups were hydrolyzed with 6 mol/L hydrochloric acid and treated as described previously [23]. Hydroxyproline values were then converted to collagen content by multiplying by a factor of 6.94 [24], while collagen concentration was calculated by dividing the collagen content by the tissue dry weight.

Determination of collagen types in the kidney

The cortex and medulla of 9-month-old RLX+/+ and RLX−/− male mice ($N = 4$ per genotype) were separated and finely diced in the presence of liquid nitrogen, and the newly synthesized and newly cross-linked collagen was extracted with 0.5 mol/L acetic acid for 24 hours at 4°C [25]. Samples were centrifuged at 13,000 rpm for 30 minutes and the acetic acid supernatant (containing the soluble collagen) discarded, while the remaining pellet, containing the maturely cross-linked matrix collagens were freeze-dried, weighed, and subjected to limited pepsin digestion (enzyme:subtrate ratio, 1:10) for 24 hours at 4°C [23]. The pepsin-digested (collagen) supernatants were collected after centrifugation (as above), freeze-dried, and dissolved in sample loading buffer, as used before [23].

The collagen chains were analyzed on 5% (wt/vol) acrylamide gels with a stacking gel of 3.5% (wt/vol) acrylamide. Interrupted electrophoresis with delayed reduction of the type III collagen disulphide bonds was used to separate the α1(III) chains from the α1(I) collagen chains [26]. The gels were stained overnight at 4°C with 0.1% (wt/vol) Coomassie brilliant blue R-250 and destained as described previously [23].

Histology of kidney tissues

Fixed kidney tissues from 9-month-old male RLX+/+ and RLX−/− mice ($N = 4$ to 6 per genotype) were washed in 70% ethanol before being processed, paraffin embedded, and cut (4 μm sections) using an AO Spencer 820 microtome. Serial sections from each tissue were stained with hematoxylin and eosin (H&E) to observe tissue structure/organization and for collagen, with the Masson trichrome stain. The stained slides were viewed using a DMRB/E microscope (Leica Microsystems, Gladesville, NSW, Australia), the images captured using a reverse transcription (RT) slider SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and stored for retrieval and analysis. Several tissue sections were screened with each stain and a representative slide chosen for figure presentation.

Morphometric evaluation of glomerular pathology

Point counting methodologies were used to quantify glomerular matrix and cells in Masson trichrome–stained sections. The technique is based on the principle that points distributed in an independent way onto a given tissue will hit different tissue compartments according to the relative extent of each compartment.

Sections were examined using a 20× objective lens combined with an eye piece graticule with ten equidistant intersecting lines, or points. For the purposes of analysis, the glomerulus was defined as matrix, cells, capillary loops, and space surrounding glomerular segments. The relative portion occupied by solid material (cells and matrix) was calculated from the number of points falling on solid material, divided by the total number of points falling on each glomerulus. Results from approximately 15 glomeruli, from each tissue section (per mouse) were expressed as the percentage fractional area (%FA). Sections from four RLX+/+ mice and six RLX−/− mice were used for evaluation.

RT-polymerase chain reaction (PCR) analysis of relaxin and relaxin receptor expression in the kidney

For RNA extraction, the kidney cortex and medulla from 1-month-old and 9-month-old RLX+/+ and RLX−/− male mice ($N = 2$ per age group and genotype) were treated as described elsewhere [19].

RT-PCR was used to determine relaxin-1, relaxin-3, and LGR7 gene expression in the cortex and medulla of 1-month-old and 9-month-old male RLX+/+ and RLX−/− mice. Fifty microliter reactions containing 100 ng of primers and 0.5 to 1 μg of the cDNA template were used for all PCR reactions. All primers used were designed to span intron-exon junctions and hence control for genomic DNA contamination. Tissues were screened for mouse relaxin-1 and relaxin-3 mRNA expression using primers previously described [15]. Tissues were also screened for mouse LGR7 expression using previously published primer sequences [18]. The LGR7 primers used in these studies were kindly provided by Mr. Daniel Scott and Dr. Ross Bathgate (Howard Florey Institute).

For relaxin-1 and relaxin-3 expression, PCR was performed as described previously [14], while touchdown PCR was used to detect LGR7 expression [18]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in separate PCR reactions to control for quality and equivalent loading of the cDNA. Aliquots of the PCR products were electrophoresed in 2% (wt/vol) agarose gels, stained with ethidium bromide, and photographed. PCR products were excised and sequenced for confirmation as described before [19].

Autoradiography of relaxin receptor expression in the kidney

Kidney tissue from 1-month-old and 9-month-old RLX+/+ and RLX−/− mice was dissected, slowly frozen in liquid nitrogen, and stored at −70°C. Sections (10 μm) were cut in a cryostat at −20°C and collected on precleaned slides subbed with poly-l-lysine (0.01% solution). Rat brain sections were included as a positive control. Slide-mounted sections were placed in a moist chamber (25°C) and preincubated in HEPES buffer (25 mmol/L HEPES and 300 mmol/L KCL, pH 7.2) containing phenylmethylsulphonylflouride (PMSF) (1 μmol/L) for 30 minutes. Slides were incubated with ∼100 pmol/L [^{33}P]-human gene 2 (H2Rlx) (B33) relaxin for 90 minutes [27]; B33 being the full form of H2 relaxin, containing a B-chain of 33 amino acids. Nonspecific binding was defined with H2Rlx (1 μmol/L). Slides were washed twice (10 minutes) in buffer, rinsed in distilled water, air-dried, and apposed onto film for 2 weeks. The film was developed in Kodak D-19 developer (5 minutes), Kodak stop solution (1 minute), Kodak fix solution (10 minutes), and gently rinsed in running water (15 minutes). Images were analyzed with ImageQuaNT^{TM} (version 4.1; Molecular Dynamics, Sunnyvale, CA, USA).

Isolation and analysis of glomeruli from the kidney

To determine if relaxin deficiency caused changes in protein and collagen expression in glomeruli, the glomeruli from 9-month-old RLX+/+ ($N = 6$) and RLX−/− ($N = 8$) mouse kidneys were isolated as described before [28], but with some modification. Briefly, each anesthetized mouse was perfused with $1.4 - 10^8$ Dynabeads (M-280 tosylactivated with 2.8 μ diameter) (Dynal Pty, Ltd., Oslo, Norway) (kindly provided by Dr. Siew Yeen Chai, Howard Florey Institute) in 40 mL of phosphate-buffered saline (PBS). The kidneys were removed from each animal and the renal cortex separated from the rest of the tissue, before the cortex was diced

Table 1. Total kidney wet weights of aging male and female RLX+/+ and RLX−/− mice

	Male RLX+/+ Mean ± SE (number)	Male RLX−/− Mean ± SE (number)	Female RLX+/+ Mean ± SE (number)	Female RLX−/− Mean ± SE (number)
1 month of age	0.17 ± 0.002 g (6)	0.18 ± 0.1 g (6)	0.17 ± 0.01 g (8)	0.16 ± 0.01 g (8)
6 months of age	0.25 ± 0.01 g (10)	0.30 ± 0.01 g (10)[a]	—	—
12 months of age	0.28 ± 0.01 g (20)	0.32 ± 0.01 g (16)[b]	0.18 ± 0.01 g (10)	0.20 ± 0.01 g (10)
19 months of age	—	—	0.25 ± 0.01 g (8)	0.28 ± 0.01 g (10)[b]

[a] $P < 0.01$; [b] $P < 0.05$, when compared with corresponding values from gender-matched and age-matched RLX+/+ mice.

into fine pieces. Diced cortical tissue was collagenase-treated, filtered, and washed as previously described [28]. The glomeruli, containing Dynabeads, were finally gathered with a magnetic particle concentrator (MPC) and washed, before being stored in Hank's balanced salt solution (HBSS) (500 μL) and observed under a microscope. Equal aliquots of each glomeruli sample were used for protein determination and hydroxyproline (collagen) determination.

For total protein determination, 50 μL aliquots of samples containing glomeruli (in HBSS) were analyzed with the Bio-Rad (Richmond, CA, USA) dye-based protein assay, as described by the manufacturer. The absorbance of each sample was read in a Beckman DU-64 spectrophotometer (Beckman-Coulter Pty Ltd., Sydney, NSW, Australia) at a wavelength of 595 nm. The remainder of each sample was hydrolyzed with 6 mol/L hydrochloric acid and analyzed for hydroxyproline (collagen) content, as described above.

Serum and urine analysis

To determine the functional consequences of relaxin deficiency on the kidney, serum was isolated from the blood of 9- to 12-month-old male RLX+/+ ($N = 4$) and RLX−/− ($N = 7$) mice and analyzed for creatinine in a Beckman Synchron CX-5 Clinical System (Fullerton, CA, USA). Nine-month-old male RLX+/+ ($N = 6$) and RLX−/− ($N = 6$) mice were maintained in metabolic cages for a 24-hour period to collect urine for determination of urinary protein (using the Beckman Synchron System).

Human recombinant relaxin treatment of relaxin-deficient mice

Twelve-month-old male RLX−/− mice ($N = 13$) were anesthetized and subjected to subcutaneous implantation of osmotic minipumps (model 2002) (Alza, Cupertino, CA, USA) as described previously [19]. The osmotic minipumps were loaded with either a 10 mmol/L citrate buffer, pH 5.0 (vehicle; $N = 6$) or 0.5 mg/kg/day rH2 (in citrate buffer; $N = 7$), which were maintained for 14 days. The dose of rH2 added was previously shown to produce circulating levels of 20 to 40 ng/mL after 14 days of treatment and successfully treat pulmonary fibrosis in 9-month-old and 12-month-old, male RLX−/− mice [19]. After 14 days, the animals were euthanized, blood withdrawn by cardiac puncture, kidneys removed and separated into cortex and medulla for hydroxyproline analysis, or placed in 10% formalin for histologic analysis (as described above). Serum was isolated from blood of rH2-treated RLX−/− mice ($N = 6$) and analyzed for creatinine.

Statistical analysis

The results were analyzed using a one-way analysis of variance (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

The effects of relaxin deficiency on kidney weight, collagen content, and types

A significant increase (15% to 20%, $P < 0.05$) in kidney wet weight was measured from 6 months to 12 months of age in male RLX−/− mice, compared with age-matched tissue weights from male RLX+/+ mice (Table 1). No significant differences between kidney weights of female RLX+/+ and RLX−/− mice were measured from 1 month to 12 months of age. However, after 19 months of age, the kidney weights of female RLX−/− mice were significantly heavier (12% to 15%, $P < 0.05$) than age-matched tissues from RLX+/+ and RLX+/− mice (Table 1).

Figure 1A shows the total dry weight of the cortex (C) and medulla (M) of aging, normal, and relaxin-deficient mice. A significant increase in dry weight of the cortex (32%, $P < 0.05$) and medulla (15%, $P < 0.05$) was measured in male RLX−/− mice at 6 months of age, compared to age-matched tissue weights from male RLX+/+ mice. The increased kidney dry weight of RLX−/− animals was only sustained in the cortex (41% increase, $P < 0.01$) of 12-month-old mice (Fig. 1A), which correlated with a 20% increase ($P < 0.05$) in kidney weight/body weight in RLX−/− mice, as compared to the same ratio in RLX+/+ mice. In female animals, no significant differences in dry weight were measured in the

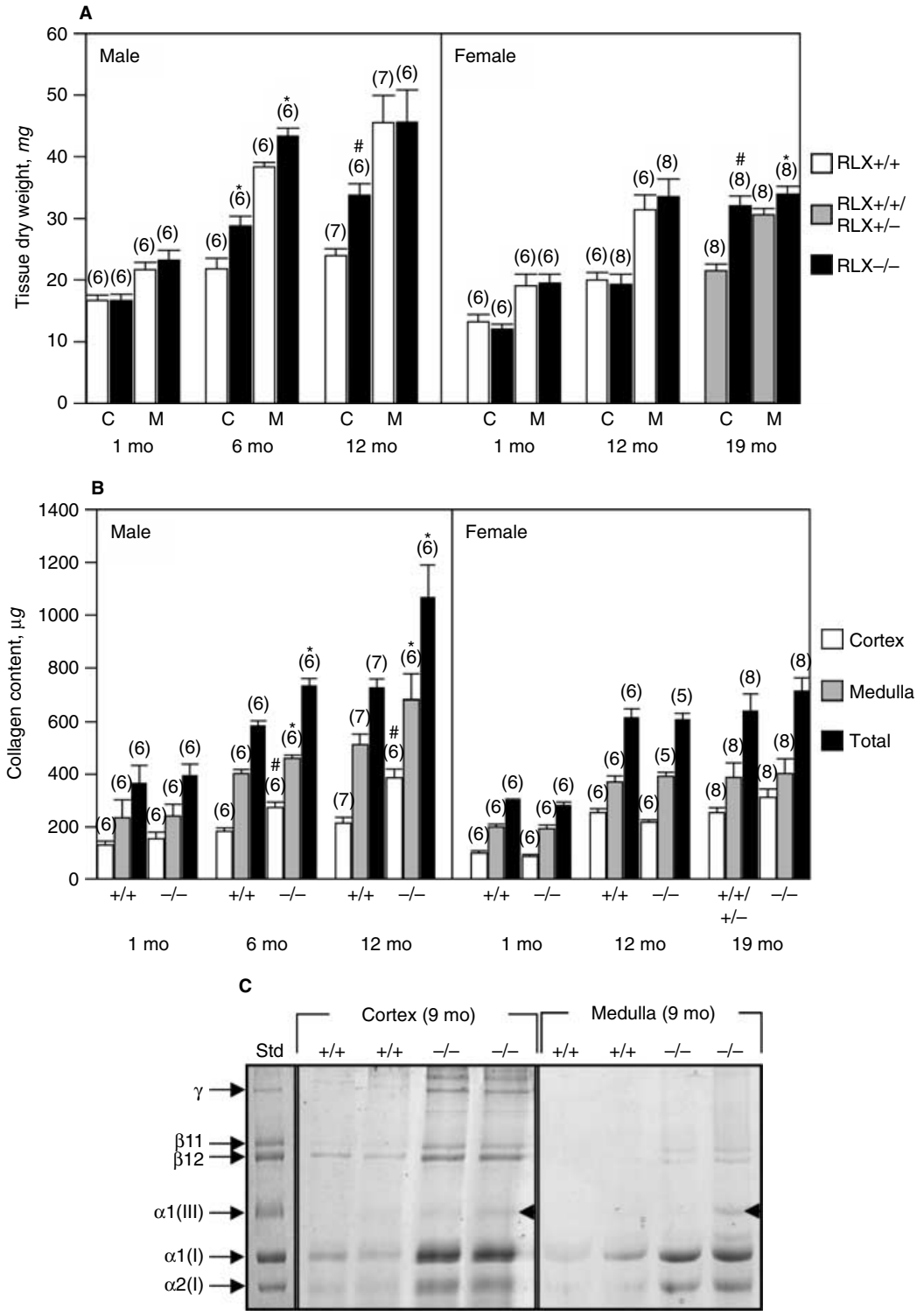

Fig. 1. Effects of relaxin deficiency on kidney dry weight (*A*), collagen content (*B*), and collagen types (*C*) in aging mice. The dry weight of the separated kidney cortex (C) and medulla (M) of male RLX+/+ and RLX−/− mice (from 1 month to 12 months of age) and female mice (from 1month to 19 months of age) was measured, along with the total collagen content from the same tissues at each age group. Total collagen content was derived from the sum of collagen in the cortex and medulla. The numbers in parenthesis represent number of samples. $^{*}P < 0.05$ and $^{\#}P < 0.01$, when compared with corresponding values from age-matched RLX+/+ mice. The pepsin-digested collagen, which shows the maturely cross-linked insoluble collagen types was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using delayed reduction of the disulfide bonds with 10% β-mercaptoethanol. The samples consist of a type I/III collagen standard from human dermis and the collagen from two individual pepsin digests from the cortex and medulla of RLX+/+ and RLX−/− mice, respectively. Four RLX+/+ and four RLX−/− mouse cortex and medulla extracts were analyzed by SDS-PAGE and gave identical results.

corresponding tissues of RLX+/+ and RLX−/− mice, at either 1 month or 12-months of age. As with the wet weight measurements (Table 1), only after 19 months of age, did we measure significant increases in cortex (45%, $P < 0.01$) and medulla (11%, $P < 0.05$) dry weight of female RLX−/− mice, compared to combined age-matched tissues from RLX+/+ and RLX+/− mice (Fig. 1A). Previous studies have shown that tissues from RLX+/− mice are similar in weight to those obtained from RLX+/+ mice (when derived from the C57Blk6Jx129SV strain) [16, 17, 19].

Similar to the pattern of dry weight, a significant elevation in total collagen content (25.5%, $P < 0.05$) was measured by marked increases in cortical (49.5%, $P < 0.01$) and medulla (15%, $P < 0.05$) collagen in 6-month-old male RLX−/− male mice, compared to measurements from age-matched RLX+/+ mice (Fig. 1B). A substantial increment in collagen content was measured in 12-month-old RLX−/− male mice, in the cortex (80.3%, $P < 0.01$) and medulla (33.4%, $P < 0.05$), resulting in a 47.2% ($P < 0.05$) increase in total collagen (Fig. 1B), compared to values obtained from age-matched RLX+/+ mice. The progressive increase in kidney collagen content of male RLX−/− mice corresponded to a significant increase in collagen concentration (collagen content as a percentage of the dry weight tissue) by 12 months of age (cortex 21.3%, $P < 0.05$; medulla 29.8%, $P < 0.05$). In female mice, a progressive increase in cortex, medulla, and total collagen content were measured with age. However, there were no significant differences in collagen content between corresponding tissues of RLX+/+ and RLX−/− female mice, at either age group (1 month, 12 months, and 19 months) studied (Fig. 1B).

Type I collagen was the predominant form of mature collagen in both the cortex and medulla of 9-month-old male RLX+/+ and RLX−/− mice, as identified by the α1(I) and α2(I) subunits (Fig. 1C). A marked increase in type I collagen monomers (α1(I) and α2(I) subunits), type I collagen dimers (β11:dimers of two α1(I) subunits; β12: dimers of α1(I) and α2(I) monomers) and collagen trimers (γ) were observed in renal extracts of RLX−/− male mice, compared to collagen I levels in RLX+/+ tissue extracts. Trace amounts of type III collagen [α1(III)] subunits were also detected in cortex and medulla extracts from RLX−/− male mice, but not from RLX+/+ mouse tissues (Fig. 1C), suggesting that kidney extracts of RLX−/− mice were also associated with a small increase in type III collagen. Types IV and V collagen were not examined in either tissue by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The effects of relaxin deficiency on kidney histology

Histologically, whole kidney tissue sections from RLX−/− mice (Fig. 2B) were larger and contained larger/thickened cortices, than tissue sections from RLX+/+ mice (Fig. 2A). These findings were consistent with the larger kidney weights from RLX−/− mice (Table 1). Little ECM (and collagen) was observed in the interstitial space (Fig. 2C) and within glomeruli (Fig. 2E) of kidneys from male RLX+/+ mice. However, kidney tissues from RLX−/− mice were associated with focal increases in interstitial matrix (collagen) deposition (Fig. 2D) and a significant ($P < 0.02$), diffuse increase in the fractional area of glomerular matrix (RLX+/+ 42.5 ± 4.2%, $N = 4$; RLX−/− 64.1 ± 5%, $N = 6$) (Fig. 2F).

Analysis of relaxin and relaxin receptor mRNA expression in the kidney

Relaxin-1 mRNA expression was only detected in RLX+/+ mouse tissues, in the kidney cortex and medulla of immature (1 month) and adult (9 months) animals, as determined by RT-PCR (Fig. 3). Relaxin-3 mRNA, however, was detected in both the cortex and medulla of RLX+/+ and RLX−/− mice (Fig. 3). LGR7 gene transcripts were inconsistently identified from 3 out of 16 cDNA samples (from immature and adult RLX+/+ mouse tissues only) and required 40 cycles of amplification to be detected (Fig. 3).

Autoradiography was performed on kidney sections from 1-month-old and 9-month-old male RLX+/+ and RLX −/− age-matched mice, using [^{33}P] human gene 2 (B33) relaxin. None of the kidney tissues displayed specific binding (data not shown), whereas the positive control (rat brain) showed clear specific binding to the fifth cortical layer as previously reported [27].

The effects of relaxin deficiency on glomerular protein and collagen

The glomeruli from the cortex of male RLX+/+ and RLX−/− mice were isolated and analyzed for total protein and collagen content. Glomerular extracts from RLX−/− mice ($N = 8$) yielded 42.4% ($P < 0.01$) more protein, when compared to the same volume of sample, analyzed from RLX+/+ mice ($N = 6$) (Fig. 4). A 42% increase ($P < 0.05$) in hydroxyproline (collagen) expression was also measured from the glomeruli of RLX−/− mice, compared to samples isolated from RLX+/+ animals (Fig. 4). These findings demonstrated that the increased glomerular protein measured in RLX−/− mice was caused by the increased collagen (fibrosis) associated with these cells, and confirmed the histologic findings of diffuse increased glomerular matrix in relaxin-deficient mice.

Functional consequences of relaxin deficiency on the kidney

To determine whether the observed relaxin-deficient induced changes in kidney structure and collagen were

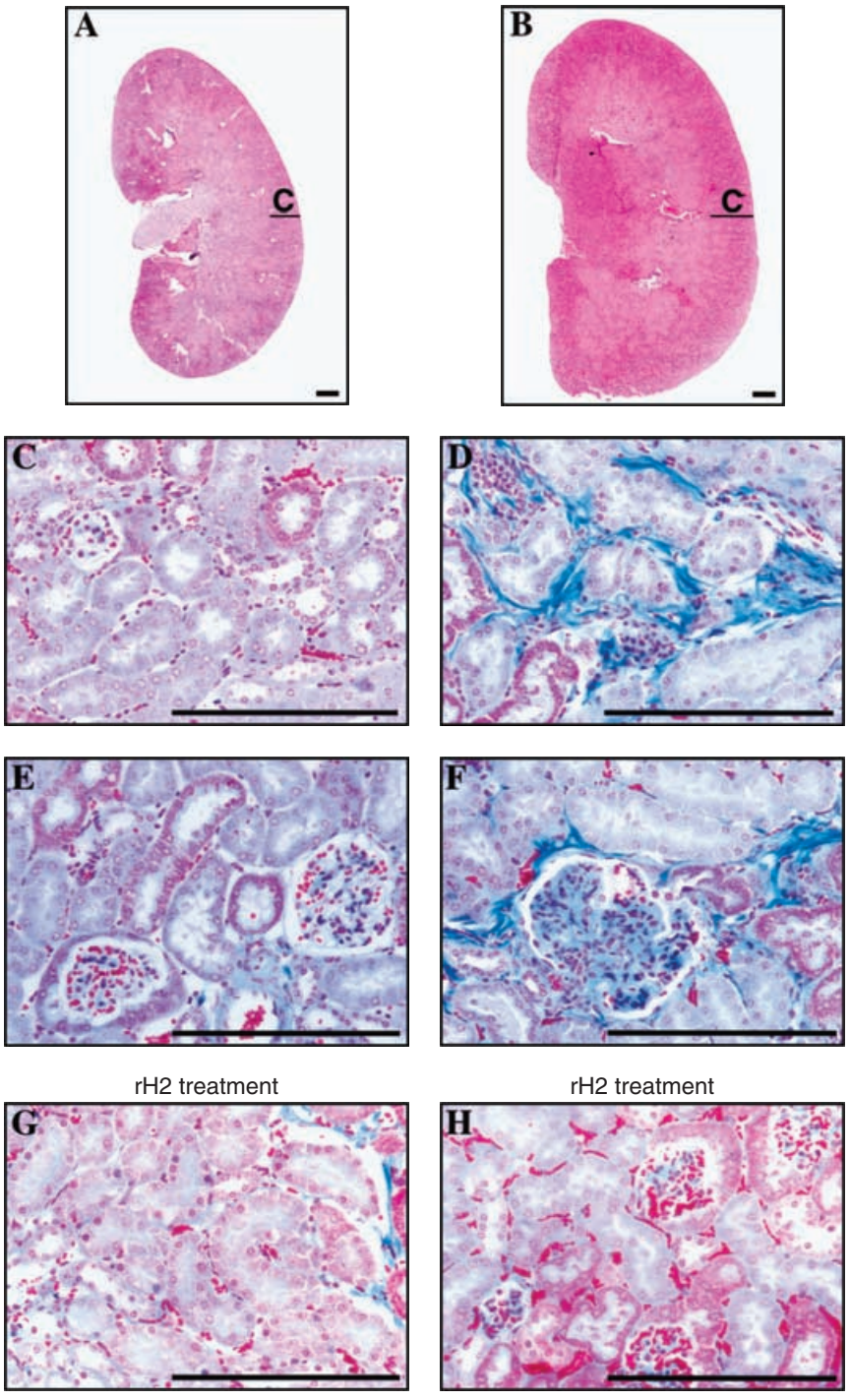

Fig. 2. Effects of relaxin deficiency on kidney histology. Hematoxylin and eosin staining of whole kidney sections from RLX+/+ (*A*) and RLX−/− (*B*) mice were performed to compare tissue size and structure. Kidney sections from RLX−/− mice were larger in size than those of RLX+/+ mice and contained thicker cortices. Masson trichrome staining was used to identify matrix (collagen) within the cortical interstitium (*C* and *D*) and glomeruli (*E* and *F*) of RLX+/+ and RLX−/− mice, respectively. Quantitative analysis of staining indicated focal increases in interstitial matrix (D) and diffuse increased glomerular matrix (F) in RLX−/− mice. rH2-treatment of RLX−/− mice resulted in a marked decreased in collagen staining within the interstitial tubules (*G*) and glomeruli (*H*), as compared to staining observed in untreated (D and F) and vehicle alone–treated mouse kidney sections.

associated with changes in kidney function of RLX−/− mice, serum creatinine and total urine protein were determined in normal and relaxin-deficient mice. Serum creatinine was modestly, but significantly ($P < 0.05$) increased in 9- to 12-month-old RLX−/− mice (20.9 ± 1.0 mmol/L; $N = 7$), compared with levels measured in RLX+/+ mice (14.5 ± 3.2 mmol/L; $N = 4$) (Fig. 5). Total urinary protein secretion from RLX−/− mice (17.4 ± 1.8 mg/24 hours; $N = 6$) was also significantly ($P < 0.05$) increased, compared with levels measured in RLX+/+ mice (11.2 ± 1.1 mg/24 hours; $N = 6$) (Fig. 5).

Reversal of renal fibrosis by recombinant human relaxin treatment

The administration of rH2 to 12-month-old, male RLX−/− mice (with established fibrosis) significantly decreased collagen expression in their kidneys (Fig. 6). Total

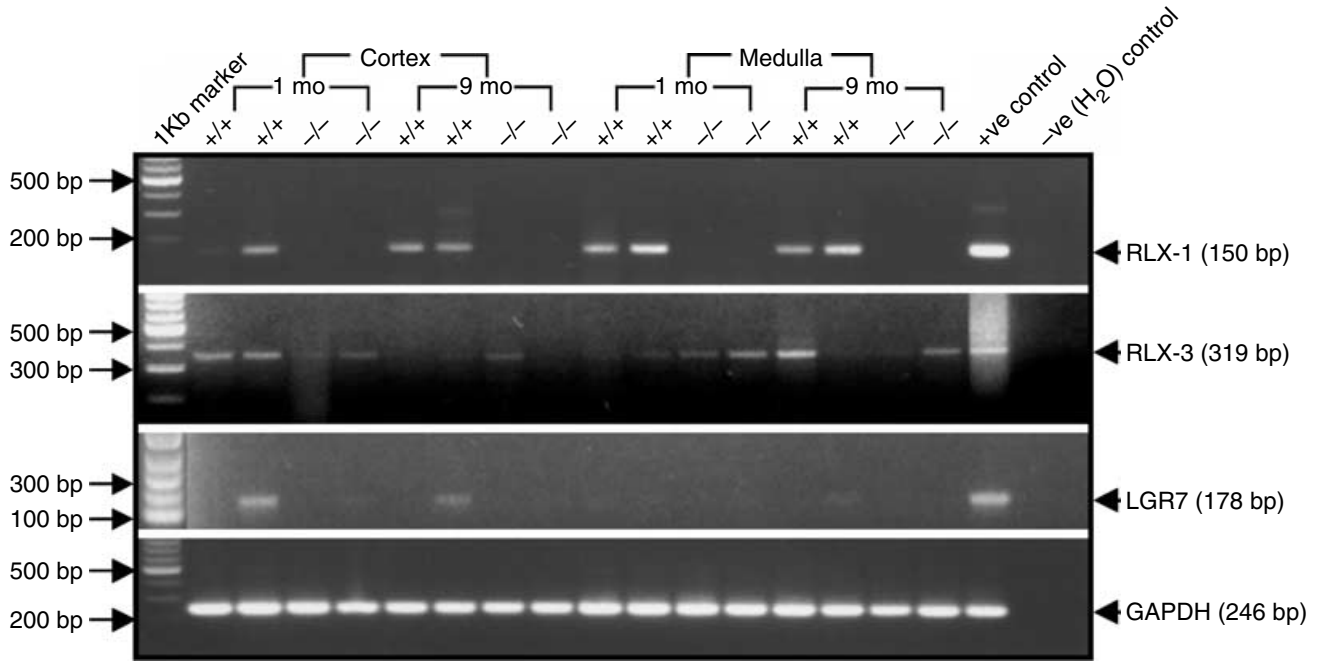

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) of relaxin-1, relaxin-3, and LGR7 mRNA expression in immature (1 month) and adult (9 months) RLX+/+ and RLX−/− mouse kidney tissues. Ethidium bromide-stained PCR products of mouse relaxin-1 (150 bp), relaxin-3 (319 bp) and LGR7 (178bp) are shown, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) products (246 bp) were used as controls for quality and equal loading of the cDNA. Samples consist of a molecular weight marker and duplicate samples from the 1-month-old cortex, 9-month-old cortex, 1-month-old medulla, and 9-month-old medulla of RLX+/+ and RLX−/− mice, respectively. cDNA from the pregnant mouse ovary was used as a positive control for relaxin-1, while mouse brain and/or cortex were used as positive controls for relaxin-3 and LGR7. Water replaced cDNA in negative control reactions for each PCR.

collagen content was inhibited in the cortex (by 62%, $P < 0.05$) and medulla (by 52%, $P < 0.05$) of RLX−/− mice, resulting in a significant reduction of total kidney collagen content (by 54%, $P < 0.05$), compared to levels measured in untreated relaxin-deficient mice. This resulted in rH2 significantly decreasing ($P < 0.05$) total kidney collagen concentration (collagen content as a percentage of the dry weight tissue) by 12%, which represented approximately half of the increased collagen concentration, measured in relaxin-deficient mice. However, the level of collagen measured after rH2 treatment of RLX−/− mice with established fibrosis, was still significantly higher ($P < 0.05$) than those observed in kidney tissues of RLX+/+ mice (Fig. 6). Histologic analysis of rH2-treated kidneys from RLX−/− mice demonstrated a decrease in tissue size and cortex size/thickness. Furthermore, rH2 treatment of RLX−/− mice decreased the level of collagen staining associated with renal interstitial tubules (Fig. 2G) and glomeruli (Fig. 2H). rH2 treatment (over 14 days) also caused a 35% to 40% decrease in serum creatinine, compared with values obtained from the serum of untreated RLX−/− mice. While this decrease in serum creatinine was not statistically significant ($P = 0.12$), these combined findings demonstrated that rH2 could be used to successfully treat fibrosis associated with renal disease.

DISCUSSION

In this study we have demonstrated for the first time that male mice, lacking the relaxin-1 gene over a lifetime developed an age-related progression of renal fibrosis. Mature male relaxin-1–deficient mice had significantly increased kidney weight and size, in addition to increased type I collagen deposition from 6 months of age and older. The increased renal collagen of RLX−/− mice led to increased renal collagen concentration by 12 months of age, and was associated with a marked thickening of the cortex, glomerulosclerosis, and, to a lesser extent, interstitial fibrosis. The increased fibrosis in RLX −/− mice was also associated with a modest reduction in kidney function. Treatment of relaxin-deficient mice with recombinant human relaxin resulted in the reversal of interstitial renal fibrosis, glomerulosclerosis, and cortical thickening, when administered to established stages of the disease. These findings confirm that relaxin is a naturally occurring inhibitor of collagen turnover during kidney development and implicate relaxin's potential as a therapeutic agent against diseases associated with or caused by renal fibrosis.

Similar to the situation in the heart of relaxin-deficient mice [20], the absence of relaxin was associated with increased fibrosis in the aging kidney of male, but not female mice. In our previous studies we had also shown that

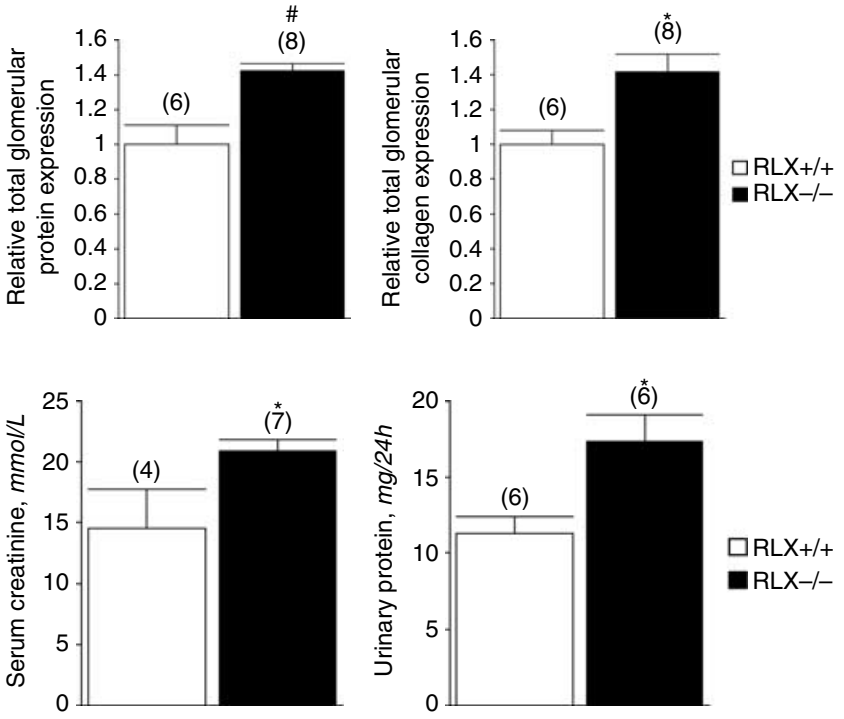

Fig. 4. Effects of relaxin deficiency on total glomerular protein and collagen expression. The glomeruli were isolated from kidney tissues of RLX+/+ and RLX−/− mice and analyzed for differences in protein content and hydroxyproline (collagen) content. Glomerular protein and collagen expression in RLX−/− mouse samples was expressed as a ratio of protein or collagen in RLX+/+ mouse tissues, respectively, which was always expressed as 1. The numbers in parenthesis represent number of sample analyzed. $^{*}P < 0.05$; $^{\#}P < 0.01$, when compared with corresponding values from RLX+/+ mice.

Fig. 5. Effects of relaxin deficiency on serum creatinine and total urine protein. To determine if the effects of relaxin deficiency on kidney structure and collagen were associated with changes in kidney function, serum creatinine and total urinary protein excretion (over a 24-hour period) were measured in RLX+/+ and RLX−/− mice. Numbers in parenthesis represent number of samples used in each assay. $^{*}P < 0.05$, when compared with corresponding values from age-matched RLX+/+ mice.

the lung of male mice developed a progressive and more severe pulmonary fibrosis, as compared with that associated with female mice [19]. These combined findings suggest that the progression of fibrosis is affected by gender and that male RLX−/− mice represent more of a relaxin "knockout" model, with no major compensatory factors that appear to replace the loss of relaxin. This is consistent with a meta-analytic report, which indicated that men are associated with a more rapid rate of chronic renal disease and show a more rapid decline in renal function with time than do women [29]. Female RLX−/− mice may be protected to a certain extent by other female-specific hormones or factors, such as estrogen, that may also compensate for the absence of relaxin in aging knockout mice. This is consistent with previous studies demonstrating that estradiol decreases collagen synthesis via activation of a mitogen-activated protein (MAP) kinase cascade [30] and reverses TGF-β1–induced cell apoptosis by a casein kinase-2–dependent mechanism [31] in renal tissues and cells. The renal protective effects of estrogen in females also include increased matrix metalloproteinase expression and reduced progression of glomerulosclerosis [32]. Thus, it appears that some tissues, such as the kidney, are more susceptible to influence by gender-specific hormones/factors, which, in turn, may prevent these tissues from the onset of fibrosis, caused by relaxin deficiency.

The effects of relaxin on the kidney have become more prevalent in recent times. In addition to being established as a mediator of renal vasodilation, hyperfiltration, and osmoregulatory changes associated with pregnancy and when administered to nonpregnant mammals [33–35], the peptide hormone has also recently been implicated as a potential antifibrotic therapy for renal disease [12, 13, 36]. Relaxin administration decreased interstitial fibrosis and restored renal function, when applied to a bromoethylamine-induced model of chronic papillary necrosis [12]. Relaxin also restored renal function when applied to models of renal mass reduction, produced by either infarction or surgical excision [13]. Our data extend those observations by demonstrating that the deletion of the relaxin gene, known to produce a peptide that regulates collagen turnover, resulted in increased long-term deposition and accumulation of collagen in the aging kidney, that was similar to levels observed by short-term renal injury [12, 13, 36]. While the whole kidney of male RLX−/− was affected by fibrosis, the most prominent changes in collagen expression were detected in the cortex. Furthermore, the long-term progression of renal fibrosis was consistent with decreased kidney function in relaxin-deficient mice. Other studies have shown that the normal glomerulus predominantly synthesizes type IV collagen [37]. During renal injury or sclerosis, cells change their phenotype, resulting in a marked increase in fibronectin expression [36] and a moderate increase in type I collagen [37] in the glomerulus. This is consistent with the biochemical and morphometric findings of our study, which demonstrated increased glomerular matrix (type I collagen) in the cortex of RLX−/− mice. Thus, our findings demonstrate that relaxin is a natural regulator of type I collagen in the developing kidney.

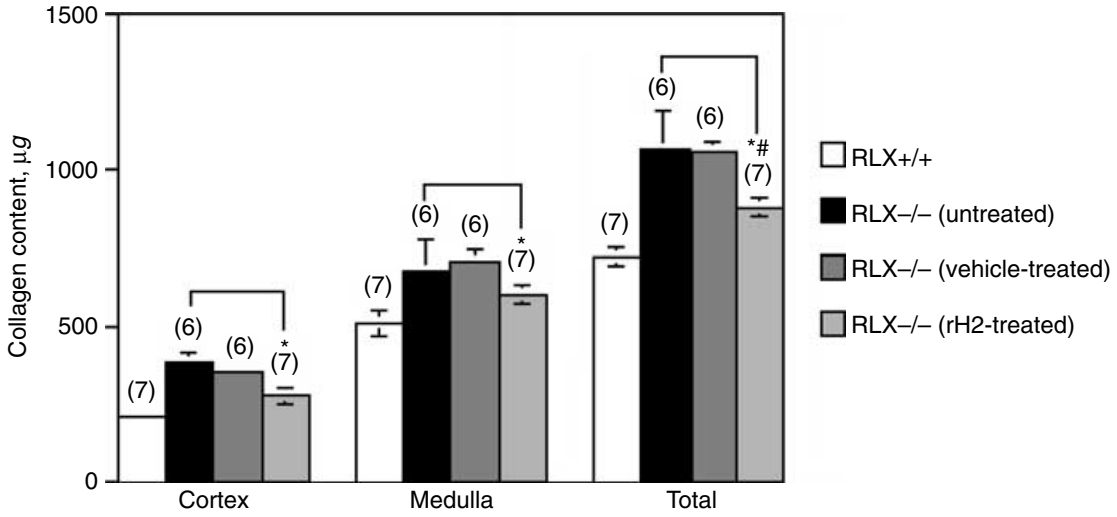

Fig. 6. Effects of rH2 treatment on the kidney of RLX-deficient mice. Total collagen content from the kidney cortex and medulla of 12-month-old RLX+/+ mice, untreated RLX−/− mice, vehicle alone–treated RLX−/− mice, and 0.5 mg/kg/day rH2-treated RLX−/− mice is shown. Total collagen content was derived from the sum of collagen content in the cortex and medulla of each tissue. Numbers in parenthesis represent number of mice used from each group. $^{*}P < 0.05$ whencompared with aged-matched untreated and vehicle-treated RLX−/− mouse collagen levels; $^{\#}P < 0.05$ when compared with total collagen from age-matched RLX+/+ mouse kidneys.

Importantly, relaxin treatment of RLX−/− mice with established fibrosis resulted in a significantly decreased (50% to 60%) total kidney collagen content, reflected by decreased collagen deposition in the cortex and medulla. As a result, relaxin treatment of RLX−/− mice decreased the level of glomerulosclerosis and interstitial fibrosis, observed in untreated animals and somewhat decreased serum creatinine, consistent with its ability to increase serum creatinine clearance and restore kidney function over longer-term treatment periods in other models of fibrosis [12]. Although collagen levels in relaxin-treated mice with established renal fibrosis were not fully restored to that observed in normal RLX+/+ mice, these findings confirm our previous studies on the lung of RLX−/−mice [19]. They also confirm that relaxin is a potent modulator of collagen overexpression, associated with renal disease.

Consistent with our findings in the lung [19], we demonstrated relaxin-1 and relaxin-3 mRNA expression in the ageing male RLX+/+ and RLX−/− mouse kidney, while there was no consistent evidence for the presence of LGR7 gene transcripts. RT-PCR analysis confirmed that relaxin-1 was only present in normal (RLX+/+) animals, while relaxin-3 was present in both immature and adult RLX+/+ and RLX−/− mice. Preliminary studies of female RLX+/+ mouse tissues has also demonstrated relaxin (^{33}P-H2Rlx) binding in highly localized regions within the kidney cortex and medulla (Dr. Tanya Burazin, unpublished data), suggesting that relaxin of renal origin may contribute to its protective effects in the kidney. However, given the multiple abnormalities in the phenotype of RLX−/− mice [16–20], it is also plausible that other relaxins of systemic/extrarenal origin may contribute to protective effects in the kidney. Since changes associated with renal fibrosis were detected in the presence of relaxin-3, it is unlikely that relaxin-3 has a direct role in the regulation of collagen in the developing and adult kidney. We found no conclusive evidence that LGR7 was present in the immature or adult kidney, perhaps suggesting that LGR7 is expressed in specific cell types or blood vessels, which may cause its expression to be low or inconsistent when whole tissue is analyzed and explain why relaxin receptor expression was not detected by autoradiography. Alternatively, relaxin binding to receptors located elsewhere may in turn affect the kidney. Preliminary data from our group suggests that LGR7 is weakly expressed in late passage primary rat mesangial cells (Dr. Ping Fu, unpublished data), which is consistent with our initial hypothesis. Mesangial cells are found in glomeruli, which, in turn, represent approximately 7% of the total cortex. Therefore, the interaction between mesangial cells and the collagen-producing fibroblasts may represent one way in which relaxin exerts its biological actions in the mouse kidney. Separate data from our group have also demonstrated that relaxin does not activate the LGR8 receptor in rodents (Dr. Ross Bathgate, unpublished data), indicating that relaxin does not mediate its effects in the mouse kidney, via LGR8. Thus, further work is still required to quantitate LGR7 receptor numbers in RLX+/+ and RLX−/− mice, to determine specific cell types that express relaxin receptors, and to determine which effects of relaxin are mediated through LGR7. The inability to detect relaxin binding sites in the mouse kidney is in accord with previous findings in a rat kidney model of fibrosis [12], even though relaxin administration in that model reduced several

markers for fibrosis and improved glomerular filtration rate.

CONCLUSION

We have demonstrated that the removal of the relaxin-1 gene from mice resulted in a build-up of collagen in the kidney, which was associated with glomerulosclerosis, modest interstitial fibrosis and a modest but significant decrease in renal function. Relaxin treatment of RLX−/− mice with established renal fibrosis caused a significant reduction in renal collagen deposition and somewhat restored renal function in RLX−/− mice. Thus, relaxin may provide an important means to regulate excessive collagen deposition in kidney diseases associated with or characterized by fibrosis.

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Reprint requests to Chrishan S. Samuel, Ph.D., Howard Florey Institute of Experimental Physiology and Medicine, The University of Melbourne, Parkville, Victoria 3010, Australia.
E-mail: c.samuel@hfi.unimelb.edu.au

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