

Relaxin Modulates Cardiac Fibroblast Proliferation, Differentiation, and Collagen Production and Reverses Cardiac Fibrosis *in Vivo*

CHRISHAN S. SAMUEL, ELAINE N. UNEMORI, ISHANEE MOOKERJEE, ROSS A. D. BATHGATE, SHARON L. LAYFIELD, JOHN MAK, GEOFFREY W. TREGEAR, AND XIAO-JUN DU

Howard Florey Institute of Experimental Physiology and Medicine (C.S.S., I.M., R.A.D.B., S.L.L., G.W.T.), University of Melbourne, Victoria 3010, Australia; Connetics Corporation (E.N.U., J.M.), Palo Alto, California 94303; and Baker Heart Research Institute (X.-J.D.), Melbourne, Victoria 8008, Australia

Cardiac fibrosis is a key component of heart disease and involves the proliferation and differentiation of matrix-producing fibroblasts. The effects of an antifibrotic peptide hormone, relaxin, in inhibiting this process were investigated. We used rat atrial and ventricular fibroblasts, which respond to profibrotic stimuli and express the relaxin receptor (LGR7), in addition to two *in vivo* models of cardiac fibrosis. Cardiac fibroblasts, when plated at low density or stimulated with TGF- β or angiotensin II (Ang II), accelerated fibroblast differentiation into myofibroblasts, as demonstrated by significantly increased α -smooth muscle actin expression, collagen synthesis, and collagen deposition (by up to 95% with TGF- β and 40% with Ang II; all $P < 0.05$). Fibroblast proliferation was significantly increased by 10^{-8} M and 10^{-7} M Ang II (63–75%; $P < 0.01$) or 0.1–1 μ g/ml IGF-I (27–40%; $P < 0.05$). Relaxin alone had no marked effect on these parameters, but it significantly

inhibited Ang II- and IGF-I-mediated fibroblast proliferation (by 15–50%) and Ang II- and TGF- β -mediated fibroblast differentiation, as detected by decreased expression of α -smooth muscle actin (by 65–88%) and collagen (by 60–80%). Relaxin also increased matrix metalloproteinase-2 expression in the presence of TGF- β ($P < 0.01$) and Ang II ($P < 0.05$). Furthermore, relaxin decreased collagen overexpression when administered to two models of established fibrotic cardiomyopathy, one due to relaxin deficiency (by 40%; $P < 0.05$) and the other to cardiac-restricted overexpression of β_2 -adrenergic receptors (by 58%; $P < 0.01$). These coherent findings indicate that relaxin regulates fibroblast proliferation, differentiation, and collagen deposition and may have therapeutic potential in diseased states characterized by cardiac fibrosis. (*Endocrinology* 145: 4125–4133, 2004)

RELAXIN, A 6-KDA polypeptide hormone, is secreted by the ovary into the circulation in the highest amounts during pregnancy and has several functions in mammals in relation to female reproductive tract physiology (1). These include an ability to remodel the extracellular matrix (ECM), particularly in the cervix and vagina of late pregnant mammals to facilitate parturition. Relaxin also acts outside the reproductive system on TGF- β -stimulated human dermal (2) and lung (3) fibroblasts to reduce collagen I and III synthesis and increase matrix metalloproteinase (MMP) expression. Additionally, relaxin has been shown to decrease collagen accumulation in several rodent models of fibrosis (3–6). These combined studies have led to the clinical development of relaxin for the treatment of fibrosis (7). However, relaxin's ability to regulate collagen overproduction in the heart has never been investigated.

Cardiac fibrosis is a hallmark of heart disease and is the result of a variety of structural changes that occur after pathological stimuli to the cardiovascular system (8). The fibrosis

Abbreviations: β_2 -AR, β_2 -Adrenergic receptor; Ang II, angiotensin II; DMEM-FBS, DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin; ECM, extracellular matrix; MMP, matrix metalloproteinase; rhRLX, recombinant human gene-2 relaxin; α -SMA, α -smooth muscle actin; VSMC, vascular smooth muscle cells.

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is characterized by a disproportionate accumulation of fibrillar collagen that occurs after myocyte death, inflammation, enhanced workload, hypertrophy, and stimulation by a number of hormones, cytokines, and growth factors (9–12). The proximal effector cells in this process are fibroblasts, which are activated to become myofibroblasts, which produce an excessive amount of collagen in response to inflammatory mediators, such as TGF- β (11) and angiotensin II (Ang II) (12). It has also been demonstrated that Ang II induces TGF- β release from cardiac fibroblasts, and together, they act in synergistic pathways to interfere with normal structure and function of the surrounding myocardium (13–15). Despite the pathophysiological significance of fibrosis, no effective treatment strategies exist.

The role of relaxin in the heart has been demonstrated by our recent studies showing elevated collagen content, leading to increased left ventricular end-diastolic pressure and chamber stiffness in relaxin-deficient mice (16). The relevance of relaxin in human heart disease has also been indicated by a recent study showing substantial changes in myocardial and circulating levels of relaxin in patients with heart failure (17). Furthermore, relaxin was shown to modulate the effects of Ang II (17), identifying it as a potential therapeutic agent against cardiovascular disease. However, direct effects of relaxin administration on cardiac fibrosis and the potential mechanisms involved have never been demonstrated.

Based on these previous observations (2–6, 16, 17), we

propose that relaxin acts through its G protein-coupled receptor (LGR7) (18) as a collagen-repressing hormone against cardiac fibrosis. In this study, we sought to investigate relaxin's effect on cardiac fibroblast proliferation and differentiation/activation and on the synthesis and deposition of collagen stimulated by profibrotic factors, which are known to be altered in diseased states. Additionally, we evaluated the antifibrotic properties of relaxin in two independent murine models of fibrosis, the relaxin-deficient (relaxin knockout) mouse, which develops an age-related progression of cardiac fibrosis (16), and the β_2 -adrenergic receptor (β_2 -AR) transgenic mouse, a more severe model of fibrotic cardiomyopathy (19).

Materials and Methods

Materials

Relaxin [recombinant human gene-2 relaxin (rhRLX)] was kindly provided by the Connetics Corporation (Palo Alto, CA). TGF- β was obtained from Bioscientific Australia (Sydney, New South Wales, Australia), Ang II was obtained from Sigma-Aldrich (St. Louis, MO), and IGF-I was obtained from R & D Systems (Minneapolis, MN).

Animals

Neonate (1 d old) Sprague Dawley rats were used for tissue collection and subsequent cardiac cell isolation and preparation. All male relaxin wild-type and relaxin knockout mice used in this study were generated from RLX heterozygous (C57Blk6jx129SV) parents (16). The male wild-type and heterozygous β_2 -AR transgenic mice used were generated from heterozygous (C57Blk6jxSjL) parents (19). The animals were housed in a controlled environment and maintained on a 14-h light, 10-h 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 and Baker Heart Research Institute's Animal Experimental Ethics Committees, which adhere to the Australian code of practice for the care and use of laboratory animals for scientific purposes.

Cell culture

To determine the distribution of relaxin and LGR7 expression in cardiac cells, atrial myocytes and fibroblasts, ventricular myocytes and fibroblasts, and vascular smooth muscle cells (VSMC) were obtained from neonate rats using standard collagenase digestion methods. The cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μ g/ml) (DMEM-FBS). Cardiac fibroblast isolation from myocytes and subsequent preparation were performed as described previously (20). These preparations contained more than 95% cardiac fibroblasts as determined by morphological appearance and immunocytochemical staining. Fibroblasts were used between passages 2 and 4 for all studies.

Determination of collagen synthesis and deposition from cardiac fibroblasts

To study the effects of relaxin on collagen expression, atrial and ventricular fibroblasts were plated at an equal density of $1-2 \times 10^5$ cells/cm² in DMEM-FBS. To investigate the effects of relaxin on TGF- β -stimulated collagen expression, cells were then treated for 72 h with rhRLX (100 ng/ml), TGF- β (1 or 2 ng/ml), or a combination of rhRLX (100 ng/ml) and TGF- β (2 ng/ml) in serum-free DMEM supplemented with lactalbumin hydrolysate (2). To investigate the effects of relaxin on Ang II-stimulated collagen expression, fibroblasts were treated with Ang II (1×10^{-7} M and 5×10^{-7} M) or a combination of rhRLX (100 ng/ml) and Ang II (1 or 5×10^{-7} M). To study the effects of relaxin alone on low-density cells, which rapidly differentiate to myofibroblasts (21), fibroblasts were also plated at 5/mm² and grown for 7 d in the absence or presence of rhRLX (100 ng/ml). The rhRLX dose used in these experiments was based on previous studies on fibroblast cultures, whereby

at this concentration, rhRLX demonstrated maximal effects on collagen expression (2, 3). Furthermore, the dose of rhRLX used was well within physiological levels of circulating relaxin in pregnant rats, which attain 50–100 ng/ml relaxin from d 14–20 of gestation and up to 180 ng/ml during parturition (22). For measurement of collagen synthesis, collagen was biosynthetically labeled with [³H]-proline in the presence of ascorbate and β -aminopropionitrile, as previously described (2). For measurement of collagen deposition into the cell matrix, cell layers (per well) were isolated and hydrolyzed with 6 M hydrochloric acid for measurement of hydroxyproline content, as described previously (23). Hydroxyproline values were then converted to total collagen content by multiplying by a factor of 6.94 (based on hydroxyproline representing approximately 14.4% of the amino acid composition of collagen in most mammalian tissues) (24).

Determination of MMP activity from cardiac fibroblasts

To determine the effects of rhRLX on MMP expression, gelatin zymography of conditioned media was performed, as previously described (25). Briefly, the cells (1×10^5 /cm²) were treated with either TGF- β (2 ng/ml) or Ang II (10^{-7} M) in the absence or presence of rhRLX (100 ng/ml) for 72 h; the final 24 h of treatment were under serum-free conditions. Equal aliquots of the collected media were then analyzed on zymogram gels consisting of 7.5% acrylamide and 1 mg/ml gelatin, and the gels were subsequently treated as previously detailed (25). Clear bands indicated gelatinolytic activity.

Determination of cardiac fibroblast proliferation

In separate experiments, the effects of relaxin on IGF-I- or Ang II-stimulated atrial and ventricular fibroblast proliferation was investigated based on their reported abilities to stimulate fibrosis (12, 13) and potentially increase cardiac fibroblast number (26, 27). Cardiac fibroblasts were plated at an equal density of 10^4 cells/96-well plate in DMEM-FBS. Twenty-four hours later, media were changed to DMEM supplemented with lactalbumin hydrolysate, and cells were labeled with [³H]-thymidine (3 μ Ci/ml) in the presence of rhRLX (100 ng/ml) alone or in combination with IGF-I (0.1 μ g/ml, 0.5 μ g/ml, and 1 μ g/ml) or Ang II (10^{-8} M and 10^{-7} M) for 72 h. [³H]-thymidine incorporation and cell counts, using a hemocytometer, were evaluated.

Western blot analysis of α -smooth muscle actin (α -SMA)

Fibroblast differentiation into myofibroblasts was determined by α -SMA expression. Ventricular fibroblasts were plated at a density of 2×10^5 cells/12-well plate and incubated with media (0.7 ml) alone, media containing TGF- β (2 ng/ml) or TGF- β (2 ng/ml), and rhRLX (100 ng/ml) for 72 h of culture. Collected media were stored at -80 C. Cell layers were trypsinized, and the protein was extracted using Trizol reagent (according to the manufacturer's instructions; Life Technologies, Gaithersburg, MD) and analyzed by the Bio-Rad dye-binding protein assay (Bio-Rad, Richmond, CA).

Protein extracts (in 1% sodium dodecyl sulfate; 10 μ g total protein/lane) were electrophoresed under nonreducing conditions on 12.5% acrylamide gels, as previously described (28). Western blot analysis was performed with a monoclonal antibody to α -SMA (M0851, 1:1000 dilution; Dako Corp., Carpinteria, CA) overnight and a goat antimouse IgG secondary antibody (1:2000 dilution; Dako) for 2 h. Densitometry of the α -SMA bands was performed using a Bio-Rad GS710 Calibrated Imaging Densitometer and Quantity-One software (Bio-Rad).

RT-PCR analysis of relaxin and relaxin receptor expression in cardiac cells

Freshly isolated cells or cells plated in six-well plates and grown to confluency in DMEM-FBS were used for RNA extraction and analysis. RNA extraction and RT-PCR of relaxin-1 (homologous to human relaxin-2) and LGR7 gene expression in atrial myocytes and fibroblasts, ventricular myocytes and fibroblasts, and VSMCs of rats ($n = 3-4$ samples per cell type) were performed as previously described (29, 30). Rat cardiac cells were screened for relaxin-1 using primer sequences detailed previously (29). Relaxin-3 expression was also determined using specific forward (5'-TGCGGAGGCTCACGATGGCGC-3') and re-

verse (5'-ATAGCTGACAGCAGGTTGGAC-3') primers. LGR7 expression was determined using the following primers: forward, 5'-GTGTATCCTTTTCGGTGTTTAAGG-3'; and reverse, 5'-GAATAAGAATTGAGCTAGATGAC-3'. For relaxin-1 gene expression, an annealing temperature of 55 C (40 cycles) was used; for relaxin-3 expression, an annealing temperature of 58 C (40 cycles) was used; and for LGR7 expression, the following (touch-down) annealing temperatures were used: 54 C (two cycles), 52 C (two cycles), 50 C (two cycles), and 48 C (34 cycles). Glyceraldehyde-3-phosphate dehydrogenase was used in separate PCR reactions to control for quality and equivalent loading of the cDNA. Aliquots of the PCR products were electrophoresed on 2% (wt/vol) agarose gels stained with ethidium bromide and photographed.

cAMP bioassay

The ability of rhRLX to induce cAMP production by rat ventricular fibroblasts was also determined using Ang II-stimulated and unstimulated ventricular fibroblasts. Fibroblasts were plated at a density of 4×10^4 cells/cm² in DMEM-FBS and then incubated with or without Ang II (10^{-7} M) for 72 h. Before cAMP measurement, cells were preincubated for 15 min with isobutylmethylxanthine (50 μ M). rhRLX (10 nM) alone, forskolin (1 μ M) alone, or a combination of rhRLX and forskolin were then added to cells, before intracellular cAMP was assayed as previously described (30) following cell lysis at 2, 5, 10, 15, and 30 min after incubation.

Relaxin treatment *in vivo* by osmotic mini-pumps

The ability of rhRLX to reduce myocardial collagen accumulation *in vivo* was investigated in two models of cardiac fibrosis, one due to relaxin disruption (16) and the other to cardiac-restricted overexpression of β_2 -AR (19). The age at which cardiac fibrosis was already established in these animals (12 months of age in male relaxin-deficient mice and 5

months of age in male β_2 -AR transgenic mice) was chosen for treatment studies based on our previous observations (16, 19). For each model, wild-type littermates were used as controls. Osmotic mini-pumps (model 2002; Alza Corp., Cupertino, CA), loaded with either vehicle (20 mM sodium acetate buffer, pH 5.0) or rhRLX (500 μ g/kg·d) were implanted sc for 14 d, which produced circulating relaxin concentrations of 20–40 ng/ml in these animals (3, 6), which is within physiological levels of serum relaxin in pregnant rodents (1, 22). The dose of rhRLX used in these studies was identical to that used to treat pulmonary fibrosis in relaxin-deficient (6) and bleomycin-treated (3) mice. Male relaxin-null mice (n = 4 treated with vehicle, n = 4 treated with rhRLX), β_2 -AR transgenic mice (n = 8 treated with vehicle, n = 8 treated with rhRLX), and their respective wild-type mice (n = 8 for each group) were used for analysis. After 14 d, ventricular tissues were collected and used for hydroxyproline analysis, as previously described (23).

Statistical analysis

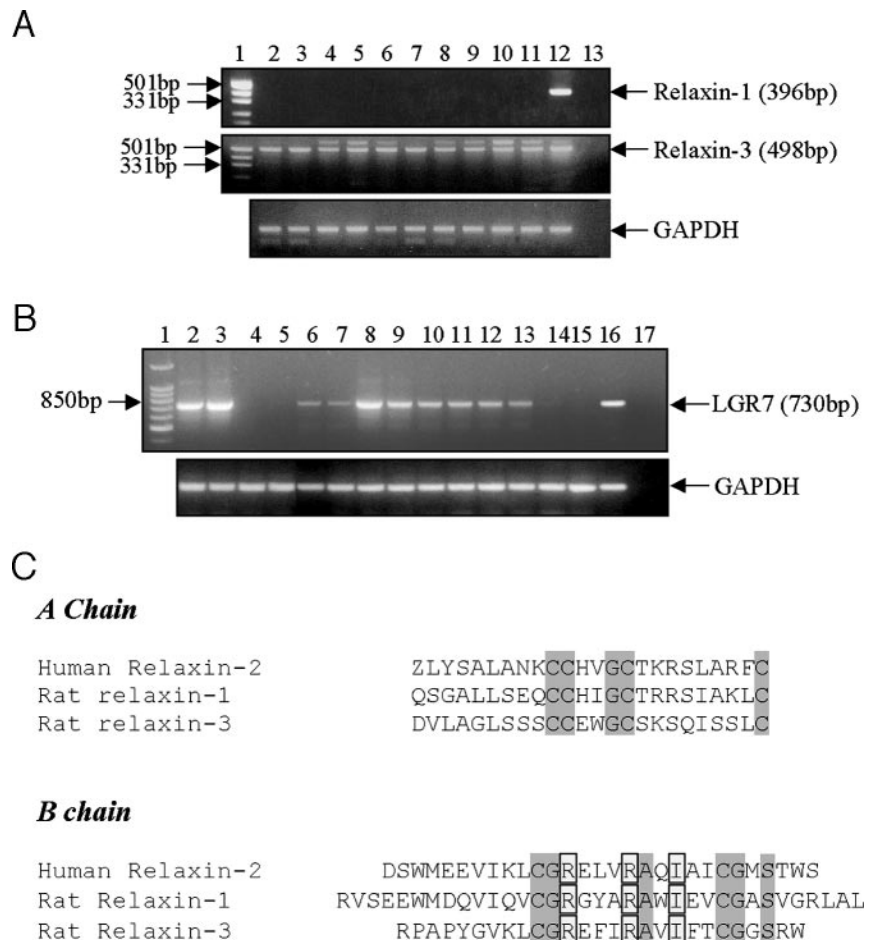
The results were analyzed using one-way ANOVA and the Newman-Keuls test for multiple comparisons between groups. All data in this article are presented as the mean \pm SEM, with $P < 0.05$ described as statistically significant.

Results

Relaxin and relaxin receptor (LGR7) mRNA expression

Relaxin-1, relaxin-3, and rat LGR7 mRNA expression from neonate atrial and ventricular myocytes and fibroblasts and VSMC was determined by RT-PCR. Rat relaxin-1 gene transcripts were undetectable in all cell types studied (Fig. 1A), which was consistent with our previous findings (29).

FIG. 1. RT-PCR of rat relaxin-1, relaxin-3, and LGR7 mRNA expression from neonatal cardiac cells. **A**, Ethidium bromide-stained PCR products of relaxin-1 (396 bp) and relaxin-3 (498 bp). Samples consist of a DNA standard (lane 1) and duplicate PCR products from atrial myocytes (lanes 2 and 3), atrial fibroblasts (lanes 4 and 5), ventricular myocytes (lanes 6 and 7), ventricular fibroblasts (lanes 8 and 9), and VSMC (lanes 10 and 11). cDNA from a late pregnant rat ovary and rat brain were used as positive controls (lane 12) for relaxin-1 and relaxin-3, respectively, whereas water replaced cDNA in negative control reactions (lane 13). **B**, Ethidium bromide-stained PCR products of LGR7 (730 bp). Samples consist of DNA standard (lane 1) and duplicate PCR products from freshly isolated atrial myocytes (lanes 2 and 3), 2-d cultured atrial myocytes (lanes 4 and 5), 2-d cultured atrial fibroblasts (lanes 6 and 7), freshly isolated ventricular myocytes (lanes 8 and 9), 2-d cultured ventricular myocytes (lanes 10 and 11), 2-d cultured ventricular fibroblasts (lanes 12 and 13), and cultured VSMC (lanes 14 and 15). cDNA from the rat brain was used as a positive control for LGR7 (lane 16), whereas water was used instead of cDNA in negative control reactions (lane 17). Glyceraldehyde-3-phosphate dehydrogenase products were used as controls for quality and equal loading of relaxin-1, relaxin-3, (A) and LGR7 (B) cDNA. **C**, Alignment of rhRLX, rat relaxin-1, and rat relaxin-3 A and B chain peptide sequences. Homologous amino acids are shaded, and the conserved relaxin receptor binding residues are boxed and shaded.



Relaxin-3 mRNA expression, however, was clearly identified in all atrial and ventricular cells studied (Fig. 1A), suggesting that it represents the predominant form of relaxin in the rat heart. LGR7 gene expression was detected in freshly isolated atrial and ventricular myocytes but was either not detected (2-d cultured atrial myocytes) or decreased (2-d cultured ventricular fibroblasts) in cultured myocytes (Fig. 1B). LGR7 was expressed in atrial and ventricular fibroblasts, and this expression persisted during culture (from passages 1–4). LGR7 transcripts were not detected in 2-d cultured VSMC (Fig. 1B). These findings demonstrated that neonatal cardiac fibroblasts expressed LGR7 and should, therefore, respond to relaxin. A comparison of the amino acid sequence of rat relaxin-1, rat relaxin-3, and rhRLX demonstrates a conserved receptor binding domain (of relaxin) that interacts with the LGR7 receptor (Fig. 1C).

Relaxin inhibits TGF- β - and Ang II-stimulated collagen deposition by reducing collagen secretion and increasing MMP activity

We then studied the ability of rhRLX to modulate collagen synthesis, degradation, and deposition by cardiac fibroblasts under a number of conditions. Atrial and ventricular fibroblasts were subjected to TGF- β or Ang II in the absence or presence of rhRLX over 3 d. TGF- β (1 ng/ml) alone induced a 85–95% increase in secretion of interstitial (types I and III) collagen into the cultured media ($P < 0.01$; Fig. 2A). Similarly, Ang II (5×10^{-7} M, 1×10^{-7} M) alone increased collagen secretion by 15–20% and 50–60% ($P < 0.05$), respectively (Fig. 2A). rhRLX (100 ng/ml) alone had no effect on collagen expression in fibroblasts, as previously described (2, 3), but significantly inhibited the TGF- β - (by 60–65%, $P < 0.05$; Fig. 2A) and Ang II- (by 70–80%, $P < 0.05$, Fig. 2A) induced effects on collagen secretion over a 72-h period.

TGF- β (2 ng/ml) treatment of cells induced a modest increase in MMP-2 expression over a 72-h period, as detected by gelatin zymography (Fig. 2B). However, rhRLX (100 ng/ml) treatment of cardiac fibroblasts in the presence of TGF- β (2 ng/ml) significantly increased both the latent (by 55–60%, $P < 0.01$) and active (by 20–25%, $P < 0.01$) forms of MMP-2, while having no marked effects on MMP-9 expression over 72-h of culture (Fig. 2B). rhRLX also increased MMP-2 expression in the presence of Ang II (by 10–30% of that from untreated cultures, $P < 0.05$) over 3 d in culture (data not shown).

Collagen deposition into the matrix of fibroblasts was also up-regulated by TGF- β (65–85%, $P < 0.05$; Fig. 2C) and Ang II (20–50%, $P < 0.05$; Fig. 2C). As with its effects of collagen secretion into the media, rhRLX (100 ng/ml) alone had no effect on collagen deposition into the matrix when applied to higher-density (atrial and ventricular) cells, but rhRLX significantly decreased collagen deposition stimulated with TGF- β ($P < 0.05$) or Ang II ($P < 0.05$). rhRLX (100 ng/ml) treatment of low-density fibroblast cultures (which accelerate the differentiation of fibroblasts to myofibroblasts) also significantly ($P < 0.05$) inhibited the collagen content of the ECM by 38% compared with the collagen content from untreated cultures (Fig. 2C).

Relaxin inhibits Ang II- and IGF-I-stimulated fibroblast proliferation

The ability of rhRLX to modulate cardiac fibroblast proliferation was investigated in the absence or presence of Ang II and IGF-I, which both potently stimulate fibroblast proliferation. Ang II significantly increased atrial and ventricular fibroblast proliferation, as determined by cell counts and [3 H]-thymidine incorporation, when administered at concentrations of 10^{-8} M (63–75%, $P < 0.01$) and 10^{-7} M (64–73%, $P < 0.01$) over a 72-h culture period (Fig. 3A). IGF-I also significantly augmented fibroblast proliferation at doses of 0.1 μ g/ml (27–30%, $P < 0.05$), 0.5 μ g/ml (36–40%, $P < 0.05$), and 1.0 μ g/ml (34–40%, $P < 0.05$) over 3 d of culture (Fig. 3B). rhRLX (100 ng/ml) alone had no significant effects on basal fibroblast proliferation but significantly inhibited the Ang II- (by 15–30%, $P < 0.05$; Fig. 3A) and IGF-I- (by 25–50%, $P < 0.05$, Fig. 3B) mediated effects on cell proliferation.

Relaxin inhibits TGF- β - and Ang II-stimulated fibroblast differentiation

Ventricular fibroblast differentiation to myofibroblasts was analyzed by measuring α -SMA expression by ventricular fibroblasts. In all experiments, an equal amount of total protein (10 μ g/sample) was analyzed. Consistent with our earlier observations, TGF- β (2 ng/ml) and Ang II (10^{-7} M) significantly increased fibroblast differentiation, as detected by Western blotting of α -SMA protein. α -SMA expression increased by 85–95% with TGF- β ($P < 0.01$) and 25–35% with Ang II ($P < 0.05$) over 72 h of culture (Fig. 4). rhRLX (100 ng/ml) significantly inhibited the TGF- β -induced effects on α -SMA expression (by 65%, $P < 0.05$). Similarly, rhRLX significantly inhibited the Ang II-mediated increase in α -SMA (by 88%, $P < 0.05$; Fig. 4).

Relaxin is not coupled to sustained increases in cAMP levels in cardiac fibroblasts

To determine whether the rhRLX-mediated signaling in cardiac fibroblasts was coupled to sustained increases in cAMP, intracellular cAMP levels were measured over a 30-min time course. Fibroblasts were either untreated or pretreated for 72 h with Ang II (10^{-7} M) before the addition of rhRLX alone (10 nM) or with forskolin (1 μ M). Treatment with forskolin induced sustained increases in cAMP over 30 min in both Ang II-treated and untreated cells. Treatment with rhRLX induced a weak and transient increase in cAMP at 2 and 5 min after stimulation but only in two of four experiments. This transient increase in cAMP was seen in both Ang II-treated and untreated cells and was much weaker in the absence of forskolin (data not shown). There was no correlation observed between cAMP responsiveness and passage number or culture conditions used.

Relaxin decreases collagen accumulation in two rodent models of cardiac fibrosis

To extend the *in vitro* findings of inhibited cardiac collagen synthesis and deposition by rhRLX, the effects of rhRLX were determined on two models of cardiac fibrosis *in vivo*. Cardiac fibrosis was established in relaxin-deficient mice by 12-

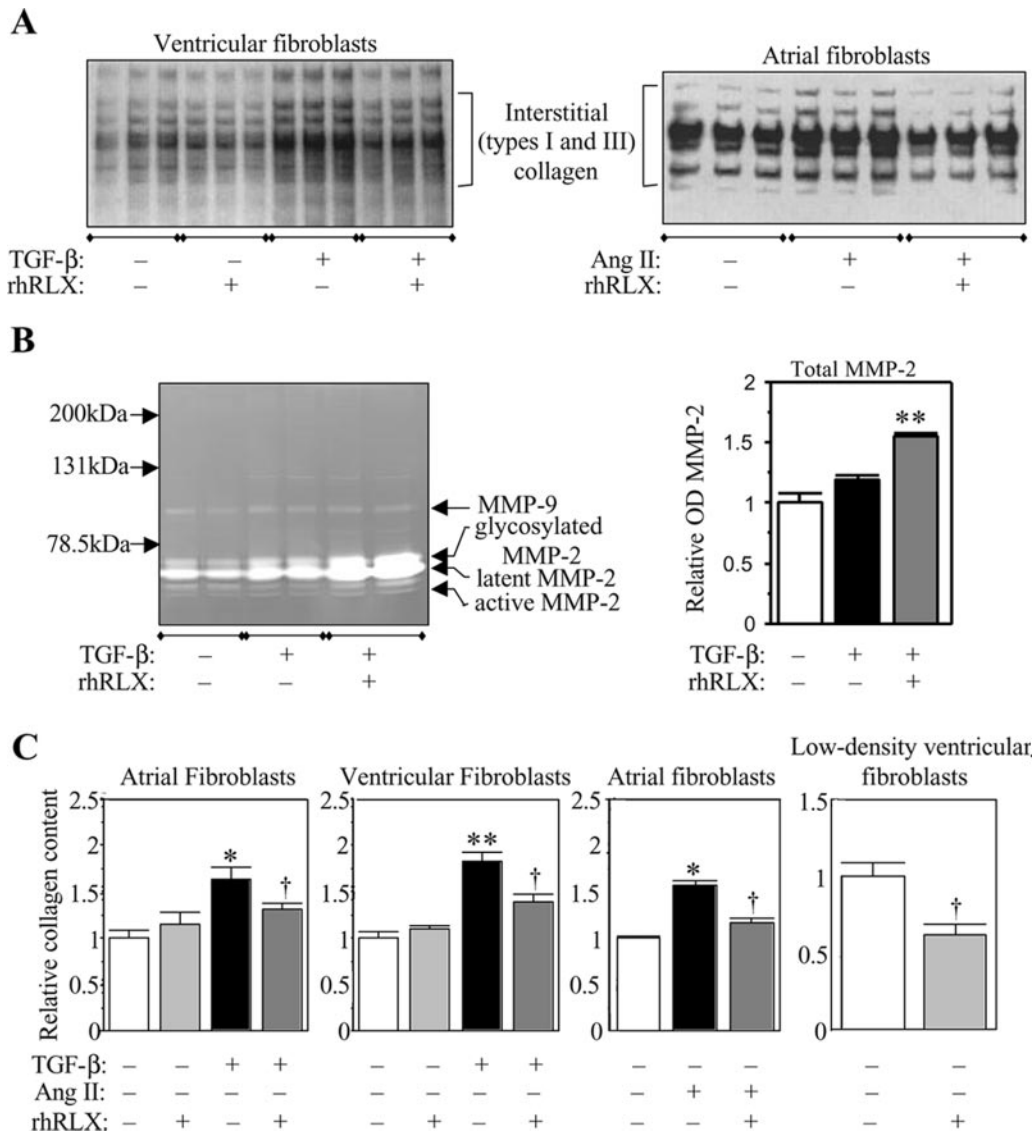


FIG. 2. Modulation of collagen synthesis, degradation, and deposition by rhRLX. A, Biosynthetically labeled interstitial collagen from untreated cardiac fibroblasts ($2 \times 10^5/\text{cm}^2$) and cells treated with either rhRLX (100 ng/ml) alone, TGF- β (1 ng/ml) alone, or TGF- β (1 ng/ml) and rhRLX (100 ng/ml), or with Ang II (5×10^{-7} M) alone or Ang II (5×10^{-7} M) and rhRLX (100 ng/ml) after 72 h of culture. Shown are representative figures of triplicate samples from three separate experiments. B, MMP-2 and MMP-9 expression and activity were determined by gelatin zymography of media from untreated cultures and cells treated with either TGF- β (2 ng/ml) or TGF- β (2 ng/ml) and rhRLX (100 ng/ml) over 72 h. Shown is a representative zymograph of duplicate samples from each group from four sets of samples per group. Also shown are the mean \pm SE of relative OD MMP-2 of the total MMP-2 (derived from the latent and active forms of MMP-2), as determined by densitometry scanning. C, Collagen content of cell layers from untreated fibroblasts and cells treated with rhRLX (100 ng/ml) alone, TGF- β (2 ng/ml) alone, or TGF- β (2 ng/ml) and rhRLX (100 ng/ml) after 72 h of culture. Also shown is the collagen content of cell layers from untreated atrial fibroblasts and cells treated with Ang II (10^{-7} M) alone or Ang II (10^{-7} M) and rhRLX (100 ng/ml) after 72 h of culture. Results are presented as the mean \pm SE of relative collagen content from three to four separate experiments. *, $P < 0.05$; and **, $P < 0.01$ compared with values from untreated cells. †, $P < 0.05$ compared with values from TGF- β - or Ang II-treated cells. Additionally, rhRLX (100 ng/ml) treatment of low-density cells ($5/\text{mm}^2$) over 7 d caused an inhibition of collagen deposition. Results are presented as the mean \pm SE of relative collagen content from three separate experiments (six assays per group from each experiment). †, $P < 0.05$ compared with values from untreated cells.

months of age, resulting in a 30% ($P < 0.05$) increase in collagen concentration (Fig. 5A). Myocardial fibrosis was more rapidly established in β_2 -AR transgenic mice at 5 months of age, as evidenced by a 60% increase ($P < 0.001$) in collagen concentration (Fig. 5B). This increment in myocardial collagen concentration over respective wild-type control values was significantly reduced by rhRLX (500 $\mu\text{g}/\text{kg}\cdot\text{d}$) treatment of animals over 14 d by 40% ($P < 0.05$) in

relaxin-deficient mice (Fig. 5A) and by 58% ($P < 0.01$) in β_2 -AR transgenic mice (Fig. 5B).

Discussion

The findings of this study demonstrated the potent antifibrotic action(s) of relaxin in cultured cardiac fibroblasts and in models of cardiac fibrosis *in vivo*. *In vitro*, rhRLX negatively

FIG. 3. Modulation of fibroblast proliferation by relaxin. **A**, Cardiac fibroblasts were stimulated with Ang II (10^{-8} M and 10^{-7} M) in the absence or presence of rhRLX (100 ng/ml) over 72 h and analyzed for cell proliferation by cell counts and [3 H]-thymidine uptake. **B**, The effects of IGF-I (0.1 μ g/ml, 0.5 μ g/ml, and 1.0 μ g/ml) on cell proliferation were also analyzed in the absence or presence of rhRLX (100 ng/ml) over 72 h. Ten samples were analyzed per group. *, $P < 0.05$; and **, $P < 0.01$ compared with values from untreated cells. †, $P < 0.05$ compared with values from Ang II- or IGF-I-treated cells.

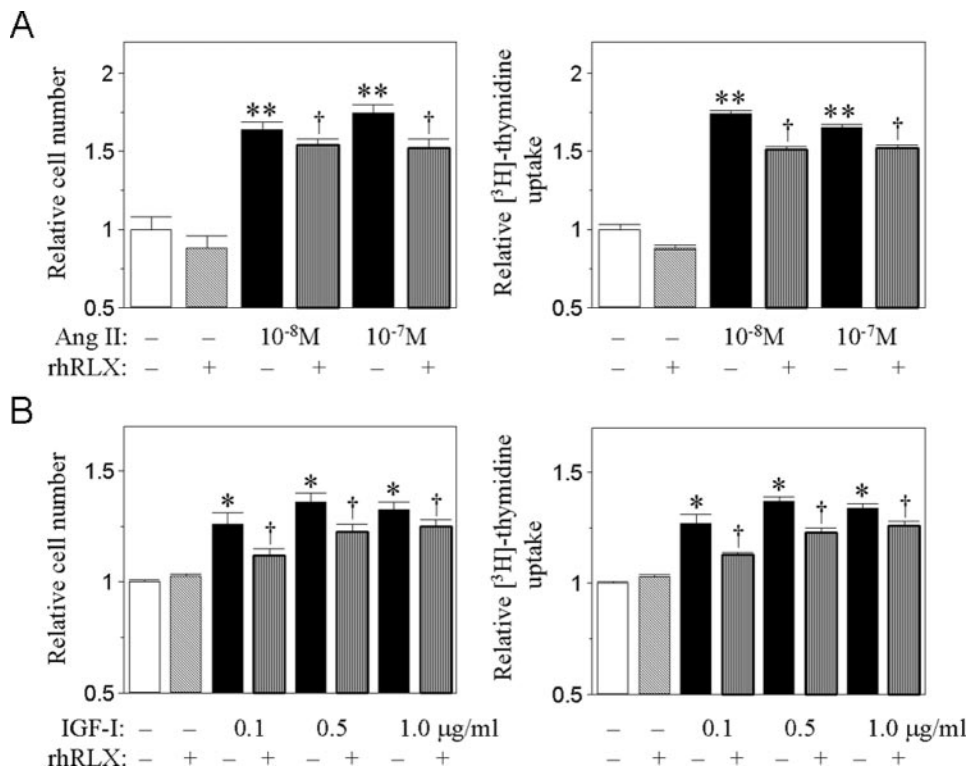
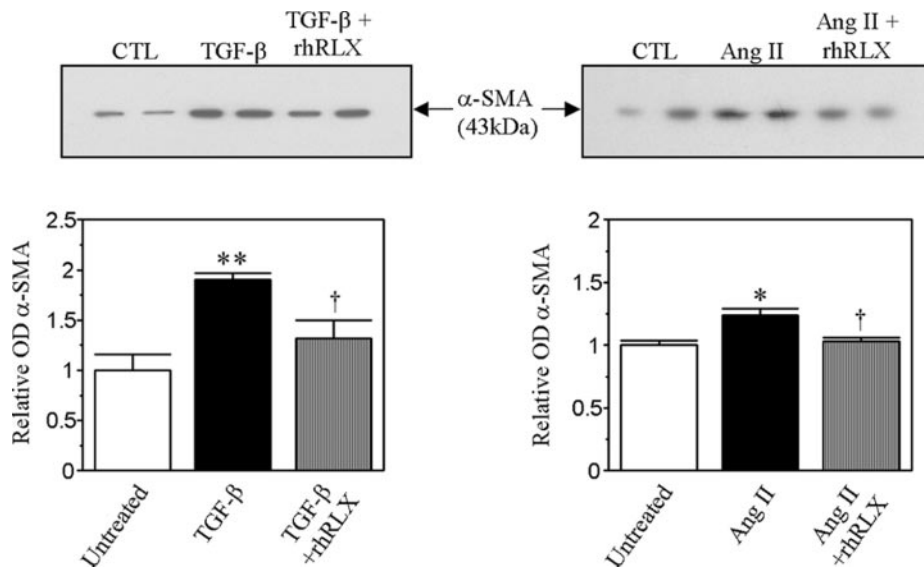


FIG. 4. Modulation of fibroblast differentiation by relaxin. Western blot analysis of α -SMA expression was used as a marker of myofibroblasts. α -SMA was sized at 43 kDa under nonreducing conditions when detected with a monoclonal antibody. In some experiments, cells were either untreated or treated with TGF- β (2 ng/ml) alone or TGF- β (2 ng/ml) and rhRLX (100 ng/ml) for 72 h. In other experiments, cells were either untreated or treated with Ang II (10^{-7} M) alone or Ang II (10^{-7} M) and rhRLX (100 ng/ml) for 72 h. Shown are representative figures of duplicate samples from three separate experiments (with either TGF- β or Ang II) and quantitation of the α -SMA products by densitometry presented as the mean \pm SE of relative OD α -SMA. *, $P < 0.05$; and **, $P < 0.01$ compared with values from untreated cells. †, $P < 0.05$ compared with values from TGF- β - or Ang II-treated cells. CTL, Control.



modulated a number of processes believed to contribute to myocardial hypertrophy, including the inhibition of fibroblast proliferation, differentiation, collagen synthesis, and collagen deposition. Furthermore, rhRLX induced a significant increase in MMP-2 expression, which most likely contributed to an increase in collagen degradation and decreased collagen deposition. rhRLX administration to two models of cardiac fibrosis also effectively lowered the collagen content in the heart within a 14-d treatment period. These combined actions of relaxin demonstrate its potential as a therapeutic agent against cardiac fibrosis.

In the normal heart, cardiac fibroblasts are relatively quiescent, but upon pathological stimuli to the heart (8–12), they

proliferate and differentiate into activated myofibroblasts. These myofibroblasts, the hallmarks of which are α -SMA expression and production of prodigious amounts of collagen, are important contributors to fibrosis and the pathology of the heart (31, 32). In the diseased heart, a number of autocrine, paracrine, and neuronal hormone systems have been found to be activated, and several of these systems, including TGF- β , Ang II, catecholamines, and endothelin-1, are known to directly stimulate fibroblast activation and collagen production (31–34). Thus, inhibitors that are able to block such profibrotic signaling are expected to have therapeutic potential against cardiac fibrosis. Interestingly, in this study, we have shown that relaxin inhibits fibroblast

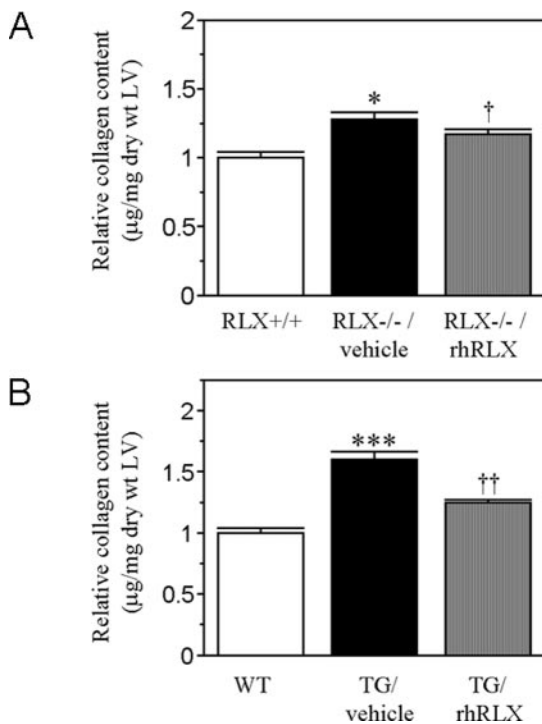


FIG. 5. Relaxin decreases collagen accumulation in two models of cardiac fibrosis *in vivo*. A, Collagen content/dry weight ventricular tissue was determined from 12-month-old relaxin wild-type (RLX^{+/+}) mice (n = 8), relaxin knockout (RLX^{-/-}) mice treated with vehicle alone (n = 4), and RLX^{-/-} mice treated with 500 µg/kg-d rhRLX (n = 4) for 14 d. B, Collagen content/dry weight ventricular tissue was also determined from 5-month-old wild-type (WT) mice (n = 8) and β_2 -AR transgenic (TG) mice treated with vehicle alone (n = 8) or with rhRLX (n = 8) for 14 d. *, $P < 0.05$; and ***, $P < 0.001$ compared with values from RLX^{+/+} and WT mice, respectively. †, $P < 0.05$ compared with values from RLX^{-/-} mice treated with vehicle alone. ††, $P < 0.01$ compared with β_2 -AR transgenic mice treated with vehicle alone. LV, Left ventricle.

activation and collagen synthesis that were stimulated by two of these factors (TGF- β and Ang II) *in vitro*, and such action was consistent with observations made *in vivo*. These findings are consistent with relaxin's ability to inhibit TGF- β - and IL-1 β -induced collagen production by human dermal and lung fibroblasts (2, 3) and with relaxin's ability to antagonize endothelin-1 activity in a number of human-derived cells (35).

The collagen-lowering effects of rhRLX observed in this study *in vivo* and *in vitro* are similar to relaxin's ability to decrease collagen expression by human dermal (2) and lung (3) fibroblasts and in other organs from animal models of fibrosis (3–6). In those studies, as well as in the current investigation, relaxin's ability to decrease fibrosis was the result of its ability to directly inhibit collagen secretion, as well as its potential ability to induce collagen degradation by increasing the release of MMPs (2–5). MMP-2 has been shown to cleave type I collagen (36), as well as fibronectin and nonfibrillar collagens. Therefore, it is likely that the deposition of a number of interstitial collagens and ECM molecules associated with fibrosis could be influenced by relaxin via its ability to induce MMP-2 activation.

Previous work has demonstrated that IGF-I, as well as Ang II, can stimulate cardiac fibroblast proliferation and may

contribute to fibrosis (26, 27). Our data suggest that relaxin's ability to inhibit organ fibrosis may also be attributable to its antiproliferative effects on fibroblasts, thus keeping in check the number of those cells that synthesize and secrete collagen. Therefore, our findings now demonstrate that relaxin may be able to modulate organ fibrosis in the following ways: 1) by inhibiting the activation of fibroblasts, as assessed by expression of α -SMA (a marker of myofibroblasts); 2) by inhibiting the proliferation of activated fibroblasts; 3) by antagonizing collagen deposition by activated fibroblasts; and 4) by increasing collagen degradation via activation of MMPs. It is of interest to note that relaxin did not influence cardiac collagen expression under basal conditions when applied over a short-term period *in vitro*. Thus, relaxin only reduced cardiac collagen synthesis and accumulation in activated cells (myofibroblasts) when stimulated by a number of factors.

Relaxin's ability to stimulate sustained increases in cAMP when applied to other cell types has been well documented (30, 37, 38), and the relaxin receptor (LGR7) has been clearly demonstrated to couple to Gs proteins and cAMP accumulation (18). However, relaxin was previously shown not to influence cAMP when applied to human uterine fibroblasts over a 15-min period (37). We investigated whether relaxin stimulation of cardiac fibroblasts was coupled to sustained increases in cAMP. Our data demonstrated that relaxin was only able to induce a weak transient rise in cAMP over a 2- to 5-min period, which returned to basal levels after 10 min, but this occurred in only two of four experiments. Therefore, relaxin stimulation is not coupled to sustained increases in cAMP in fibroblasts, which is consistent with the study on human uterine fibroblasts (37). Furthermore, the weak transient rise in cAMP in two experiments was also seen in normal fibroblasts not stimulated with Ang II; hence, the mechanism by which relaxin reverses myofibroblast phenotype is likely to be independent of cAMP signaling. Therefore, it is likely that relaxin induces its effects via other mechanisms. Indeed, relaxin has been shown to modulate a number of its effects in other target cells via the MAPK pathway (37–39) and induce MAPK phosphorylation in human uterine fibroblasts (37). The potential interactions of relaxin signaling with that of Gq-mediated activation of Ras/Raf/MAPK/ERK by Ang II (40) and activation of MAPK and ERK by TGF- β (41) are the subject of future investigation.

In this study, we used two murine models of fibrotic cardiomyopathy to test whether relaxin treatment could reverse already established fibrosis in the heart. Our data indicated that relaxin treatment was associated with a similar and significant reduction in collagen content in both models within only a 2-wk period of treatment with relaxin. It might be argued that treatment of the relaxin-null mice with exogenous relaxin would simply be replacing the hormonal deficit and that the results on cardiac fibrosis are not unexpected. However, the selection of animals at 12 months of age was made to ensure that cardiac fibrosis was already well established in these animals (16). Furthermore, this efficacy of reversing fibrosis was found using another strain of mouse in which the etiology of cardiac fibrosis is independent of relaxin (*i.e.* due to cardiac overexpression of β_2 -ARs). Although

treatment with drugs like angiotensin converting enzyme inhibitors have been reported to reduce the amount of collagens in the heart (42), such efficacy usually requires much longer periods of treatment. Thus, the significant reduction in collagen expression by short-term relaxin treatment is encouraging for this peptide as a therapeutic agent, specifically for the reversal of cardiac fibrosis.

In conclusion, we have demonstrated that relaxin inhibits cardiac collagen secretion and deposition by directly inhibiting cardiac fibroblast proliferation, differentiation, and activation and increasing MMP activity. Relaxin also markedly reversed collagen overexpression in two models of cardiac fibrosis *in vivo*. Of great interest was the ability of relaxin to induce such potent and rapid antifibrotic effects in the heart over a 2-wk period. Thus, relaxin provides an important means to regulate excessive collagen deposition in heart diseases characterized by fibrosis.

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Address all correspondence and requests for reprints to: Chrisan S. Samuel, Ph.D., Howard Florey Institute, Gate 11, University of Melbourne, Parkville, Victoria 3010, Australia. E-mail: c.samuel@hfi.unimelb.edu.au.

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Present address for E.N.U.: BAS Medical Inc., San Mateo, California 94402.

References

- Sherwood OD 1994 Relaxin. In: Knobil E, Neill JD, eds. *Physiology of reproduction*. 2nd ed. New York: Raven Press; 861–1009
- Unemori EN, Amento EP 1990 Relaxin modulates synthesis and secretion of procollagenase and collagen by human dermal fibroblasts. *J Biol Chem* 265: 10681–10685
- Unemori EN, Pickford LB, Salles AL, Piercy CE, Grove BH, Erikson ME, Amento EP 1996 Relaxin induces an extracellular matrix-degrading phenotype in human lung fibroblasts *in vitro* and inhibits lung fibrosis in a murine model *in vivo*. *J Clin Invest* 98:2739–2745
- Garber SL, Mirochnik Y, Brecklin CS, Unemori EN, Singh AK, Slobodskoy L, Grove BH, Arruda JA, Dunea G 2001 Relaxin decreases renal interstitial fibrosis and slows progression of renal disease. *Kidney Int* 59:876–882
- Williams EJ, Benyon RC, Trim N, Hadwin R, Grove BH, Arthur MJ, Unemori EN, Iredale JP 2001 Relaxin inhibits effective collagen deposition by cultured hepatic stellate cells and decreases rat liver fibrosis *in vivo*. *Gut* 49:577–583
- Samuel CS, Zhao C, Bathgate RAD, Bond CP, Burton MD, Parry LJ, Summers RJ, Tang MLK, Amento EP, Tregear GW 2003 Relaxin deficiency in mice is associated with an age-related progression of pulmonary fibrosis. *FASEB J* 17:121–123
- Erikson MS, Unemori EN 2001 Relaxin clinical trials in systemic sclerosis. In: Tregear GW, Ivell R, Bathgate RA, Wade JD, eds. *Relaxin 2000: proceedings of the 3rd international conference on relaxin and related peptides*. The Netherlands: Kluwer Academic Publishers; 373–381
- Judgutt BI 2003 Remodeling of the myocardium and potential targets in the collagen degradation and synthesis pathways. *Curr Drug Targets Cardiovasc Haematol Disord* 3:1–30
- Weber KT 1989 Cardiac interstitium in health and disease: the fibrillar collagen network. *J Am Coll Cardiol* 13:1637–1652
- Bishop JE, Lindahl G 1999 Regulation of cardiovascular collagen synthesis by mechanical load. *Cardiovasc Res* 42:27–44
- Lijnen PJ, Petrov VV, Fagard RH 2000 Induction of cardiac fibrosis by transforming growth factor- β (1). *Mol Genet Metab* 71:418–435
- Lijnen PJ, Petrov VV, Fagard RH 2000 Induction of cardiac fibrosis by angiotensin II. *Methods Find Exp Clin Pharmacol* 22:709–723
- Chua CC, Diglio CA, Siu BB, Chua BH 1994 Angiotensin II induces TGF- β 1 production in rat heart endothelial cells. *Biochim Biophys Acta* 1223:141–147
- Lee AA, Dillmann WH, McCulloch AD, Villarreal FJ 1995 Angiotensin II stimulates the autocrine production of transforming growth factor- β 1 in adult rat cardiac fibroblasts. *J Mol Cell Cardiol* 27:2347–2357
- Campbell SE, Katwa LC 1997 Angiotensin II stimulated expression of transforming growth factor- β 1 in cardiac fibroblasts and myofibroblasts. *J Mol Cell Cardiol* 29:1947–1958
- Du XJ, Samuel CS, Gao XM, Zhao L, Parry LJ, Tregear GW 2003 Increased myocardial collagen and ventricular diastolic dysfunction in relaxin deficient mice: a gender-specific phenotype. *Cardiovasc Res* 57:395–404
- Dschietzig T, Richter C, Bartsch C, Laule M, Ambruster FP, Baumann G, Stangl K 2001 The pregnancy hormone relaxin is a player in human heart failure. *FASEB J* 15:2187–2195
- Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, Hsueh AJ 2002 Activation of orphan receptors by the hormone relaxin. *Science* 295:671–674
- Gao XM, Agrotis A, Autelitano DJ, Percy E, Woodcock EA, Jennings GL, Dart AM, Du XJ 2003 Sex hormones and cardiomyopathic phenotype induced by cardiac β 2-adrenergic receptor overexpression. *Endocrinology* 144:4097–4105
- Gray MO, Long CS, Kalinyak JE, Li H-T, Karliner JS 1998 Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF- β 1 and endothelin-1 from fibroblasts. *Cardiovasc Res* 40:352–363
- Masur SK, Dewal HS, Dinh TT, Erenburg I, Petridou S 1996 Myofibroblasts differentiate from fibroblasts when plated at low density. *Proc Natl Acad Sci USA* 93:4219–4223
- Sherwood OD, Crnekovic VE, Gordon WL, Rutherford JE 1980 Radioimmunoassay of relaxin throughout pregnancy and during parturition in the rat. *Endocrinology* 107:691–698
- Samuel CS, Butkus A, Coghlan JP, Bateman JF 1996 The effect of relaxin on collagen metabolism in the nonpregnant rat pubic symphysis: the influence of estrogen and progesterone in regulating relaxin activity. *Endocrinology* 137: 3884–3890
- Gallop PM, Paz MA 1975 Posttranslational protein modifications, with special attention to collagen and elastin. *Physiol Rev* 55:418–487
- Talhok RS, Chin JR, Unemori EN, Werb Z, Bissell MJ 1991 Proteinases of the mammary gland: developmental regulation *in vivo* and vectorial secretion *in culture*. *Development* 112:439–449
- McEwan PE, Gray GA, Sherry L, Webb DJ, Kenyon CJ 1998 Differential effects of angiotensin II on cardiac proliferation and intramyocardial perivascular fibrosis *in vivo*. *Circulation* 98:2765–2773
- Van Eickels M, Vetter H, Grohe C 2000 Angiotensin-converting enzyme (ACE) inhibition attenuates insulin-growth factor-I (IGF-I) induced cardiac fibroblast proliferation. *Br J Pharmacol* 131:1592–1596
- Samuel CS, Sakai LY, Amento EP 2003 Relaxin regulates fibrillin-2, but not fibrillin-1, mRNA and protein expression by human dermal fibroblasts and murine fetal skin. *Arch Biochem Biophys* 411:47–55
- Kompa AR, Samuel CS, Summers RJ 2002 Inotropic responses to human gene 2 (B29) relaxin in a rat model of myocardial infarction (MI): effect of pertussis toxin. *Br J Pharmacol* 137:710–718
- Bathgate RAD, Samuel CS, Burazin TCD, Layfield S, Claasz AA, Reytomas IGT, Dawson NF, Zhao C, Bond C, Summers RJ, Parry LJ, Wade JD, Tregear GW 2002 Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. *J Biol Chem* 277:1148–1157
- Eghbali M 1998 Cardiac fibroblasts: function, regulation of gene expression, and phenotypic modulation. *Basic Res Cardiol* 87(Suppl 2):183–189
- Bouzeghrane F, Thibault G 2002 Is angiotensin II a proliferative factor of cardiac fibroblasts? *Cardiovasc Res* 53:304–312
- Petrov VV, Fagard RH, Lijnen PJ 2002 Stimulation of collagen production by transforming growth factor- β 1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension* 39:258–263
- Eddy AA 1996 Molecular insights into renal interstitial fibrosis. *J Am Soc Nephrol* 7:2495–2508
- Dschietzig T, Bartsch C, Richter C, Laule M, Baumann G, Stangl K 2003 Relaxin, a pregnancy hormone, is a functional antagonist of endothelin-1-mediated vasoconstriction by stimulation of endothelin type-B-receptor expression via ERK-1/2 and nuclear factor- κ B. *Circ Res* 92:32–40
- Aimes RT, Quigley JP 1995 Matrix metalloproteinase-2 is an interstitial collagenase. *J Biol Chem* 270:5872–5876

37. **Palejwala S, Stein D, Wojtczuk A, Weiss G, Goldsmith LT** 1998 Demonstration of a relaxin receptor and relaxin-stimulated tyrosine phosphorylation in human lower uterine segment fibroblasts. *Endocrinology* 139:1208–1212
38. **Bartsch O, Bartlick B, Ivell R** 2001 Relaxin signalling links tyrosine phosphorylation to phosphodiesterases and adenylyl cyclase activity. *Mol Hum Reprod* 7:799–809
39. **Zhang Q, Liu SH, Erikson M, Lewis M, Unemori E** 2002 Relaxin activates the MAP kinase pathway in human endometrial stromal cells. *J Cell Biochem* 85:536–544
40. **Tharaux PL, Chatziantoniou C, Fakhouri F, Dussaule JC** 2000 Angiotensin II activates collagen I gene through a mechanism involving the MAP/ER kinase pathway. *Hypertension* 36:330–336
41. **Funaba M, Zimmerman CM, Matthews LS** 2002 Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. *J Biol Chem* 277:41361–41368
42. **Lijnen PJ, Petrov VV** 2003 Role of intracardiac renin-angiotensin-aldosterone system in extracellular matrix remodeling. *Methods Find Exp Clin Pharmacol* 25:541–564

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