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1368 *Biochemistry* **2011,** *50,* 1368–1375
DOI: 10.1021/bi1013968

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H3 Relaxin Demonstrates Antifibrotic Properties via the RXFP1 Receptor[†]

Mohammed Akhter Hossain,[‡,§,#] Bryna Chow Suet Man,[‡,∥,#] Chongxin Zhao,[‡] Qi Xu,[⊥] Xiao-Jun Du,[⊥] John D. Wade,[‡,§] and Chrishan S. Samuel*[,‡,∥]

[‡]*Howard Florey Institute,* [§]*School of Chemistry, and* [∥]*Department of Biochemistry and Molecular Biology, The University of Melbourne, Parkville, Victoria 3010, Australia, and* [⊥]*Baker IDI Heart and Diabetes Institute, St. Kilda Road Central, Melbourne, Victoria 8008, Australia.* [#]*M.A.H. and B.C.S.M. contributed equally to the manuscript*

Received August 30, 2010; Revised Manuscript Received January 11, 2011

ABSTRACT: Human gene 3 (H3) relaxin is the most recently discovered member of the relaxin peptide family and can potentially bind all of the defined relaxin family peptide receptors (RXFP1−4). While its effects as a neuromodulator are being increasingly studied through its primary receptor, RXFP3, its actions via other RXFPs are poorly understood. Hence, we specifically determined the antifibrotic effects and mechanisms of action of H3 relaxin via the RXFP1 receptor using primary rat ventricular fibroblasts *in vitro*, which naturally express RXFP1, but not RXFP3, and a mouse model of fibrotic cardiomyopathy *in vivo*. Transforming growth factor β1 (TGF-β1) administration to ventricular fibroblasts significantly increased Smad2 phosphorylation, myofibroblast differentiation, and collagen deposition (all $p < 0.05$ vs untreated controls), while having no marked effect on matrix metalloproteinase (MMP) 9, MMP-13, tissue inhibitor of metalloproteinase (TIMP) 1, or TIMP-2 expression over 72 h. H3 relaxin (at 100 and 250 ng/mL) almost completely abrogated the TGF-β1-stimulated collagen deposition over 72 h, and its effects at 100 ng/mL were equivalent to that of the same dose of H2 relaxin. Furthermore, H3 relaxin (100 ng/mL) significantly inhibited TGF-β1-stimulated cardiac myofibroblast differentiation and TIMP-1 and TIMP-2 expression to an equivalent extent as H2 relaxin (100 ng/mL), while also inhibiting Smad2 phosphorylation to approximately half the extent of H2 relaxin (all $p < 0.05$ vs TGF-β1). Lower doses of H3 (50 ng/mL) and H2 (50 ng/mL) relaxin additively inhibited TGF-β1-stimulated collagen deposition *in vitro*, while H3 relaxin was also found to reverse left ventricular collagen overexpression in the model of fibrotic cardiomyopathy *in vivo*. These combined findings demonstrate that H3 relaxin exerts antifibrotic actions via RXFP1 and may enhance the collagen-inhibitory effects of H2 relaxin.

Three nonallelic relaxin genes have been identified in humans, which produce H1, H2,[1] and H3 relaxin, respectively (*1*). Most other mammals including rodents, however, contain only two relaxin genes, which produce relaxin (equivalent to H2 relaxin) and relaxin-3 (equivalent to H3 relaxin) (*1*). H2 relaxin (and relaxin in other species) represent the major stored and circulating forms of relaxin and have been the most investigated to date (*1*−*7*). A plethora of studies have demonstrated that H2 relaxin has several biological actions in the body, which are centered around its antiinflammatory, antifibrotic, antiapoptotic, antihypertrophic, cardioprotective, vasodilatory, and proangiogenic actions (*1*−*7*), many of which are mediated through its primary G-protein-coupled receptor, relaxin family peptide receptor 1 (RXFP1) (*8*).

On the other hand, comparatively little is known about the more recently discovered H3 relaxin (*9*, *10*) and its highly conserved species equivalent, relaxin-3 (*11*). While the distribution of H3 relaxin in humans remains unknown, anatomical studies of relaxin-3 in rodents and primates suggest that it is predominantly expressed in the brain, particularly within neurons of the nucleus insertus (*9*, *10*, *12*, *13*) in addition to nerve fibers and terminals within the cortex, hippocampus, thalamus, hypothalamus, and midbrain. Thus, H3 relaxin and relaxin-3 are thought to primarily function as neuromodulators and have been implicated in regulating arousal, feeding, learning, and memory and central responses to physiological stressors (*14*−*16*).

Studies using recombinant (*17*) and chemically synthesized (*18*) H3 relaxin have identified it as the cognate ligand for relaxin family peptide receptor 3 (RXFP3; formerly known as GPCR-135), which is also widely distributed in the brain (*12*, *13*, *17*). However, it has been demonstrated that H3 relaxin can bind to all four defined receptors of the relaxin family of peptides, including RXFP1 (*18*), RXFP2 (the primary receptor for insulin-like peptide 3) (*18*), and RXFP4 (the primary receptor for insulin-like peptide 5) (*19*, *20*), suggesting that it may have additional roles through these receptors, other than its neuromodulatory actions via RXFP3.

[†]This study was supported by a John T. Reid Charitable Trusts Fellowship to M.A.H., National Health and Medical Research Council (NHMRC) of Australia Senior Research Fellowships to X.-J.D. and J.D.W., a National Heart Foundation of Australia/NHMRC RD Wright Fellowship to C.S.S., and a NHMRC Project Grant (508995) to J.D.W. and by the Victorian Government's Operational Infrastructure Support Program.

*To whom correspondence should be addressed at the Howard Florey Institute, The University of Melbourne. Phone: + 61 3 8344 0416. Fax: +61 3 9348 1707. E-mail: chrishan.samuel@florey.edu.au.

[1]Abbreviations: AR, adrenergic receptors; H2, human gene 2; H3, human gene 3; GPCR, G-protein-coupled receptor; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MMP, matrix metalloproteinase; RP-HPLC, reverse-phase high-performance liquid chromatography; RXFP, relaxin family peptide receptor; SMA, smooth muscle actin; TGF-β1, transforming growth factor β1; TIMP, tissue inhibitor of metalloproteinase.

 Published on Web 01/13/2011 © 2011 American Chemical Society

Indeed H3 relaxin has been shown to promote matrix metalloproteinase (MMP) 2 levels in a dose-dependent manner, when administered to rat ventricular fibroblasts *in vitro*, and stimulate water drinking in rats *in vivo* (*18*) in a comparable manner to that of H2 relaxin, via a high-affinity interaction with RXFP1. These findings imply that H3 relaxin may mimic the matrix-remodeling and tissue homeostasis-related actions of H2 relaxin through the RXFP1 receptor. Consistent with the effects of H3 relaxin on MMP-2 activity *in vitro*, relaxin-3 has been found to inhibit cardiac fibrosis and improve cardiac dysfunction in an experimental rat model of cardiac toxicity *in vivo* (*21*), again suggesting that H3 relaxin/relaxin-3 may possess similar matrix-remodeling and antifibrotic actions to that of H2 relaxin via RXFP1.

On the basis of these previous observations (*18*, *21*), we have sought to determine the mechanisms by which H3 relaxin mediates its antifibrotic actions via the RXFP1 receptor in neonatal rat ventricular fibroblasts *in vitro* (which only express RXFP1 but not RXFP3) (*22*) and in a mouse model of fibrotic cardiomyopathy *in vivo* (*22*, *23*), which have both been used to evaluate the antifibrotic actions of H2 relaxin (*22*, *23*). Additionally, we evaluated if H2 and H3 relaxin had any synergistic antifibrotic actions via the RXFP1 receptor.

EXPERIMENTAL PROCEDURES

Materials. The H3 relaxin used in these studies was chemically synthesized and characterized as described previously (*18*). Recombinant H2 relaxin was kindly provided by Corthera Inc. (San Mateo, CA), and TGF-β1 was obtained from R&D Systems (Minneapolis, MN).

Animals. Neonate (1-day-old) Sprague-Dawley rats were used for tissue collection and subsequent cardiac cell isolation and preparation. The 4−5-month-old male wild-type and heterozygous β2-adrenergic receptor (β2-AR) mice used in these studies were generated from heterozygous (C57Blk6JxSJL) parents (*22*−*24*). 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. The detailed experiments were approved by the Howard Florey Institute's and Baker IDI Heart and Diabetes Institute's Animal Ethics Committees, which adhere to the Australian code of practice for the care and use of laboratory animals for scientific purposes.

Cell Culture. Ventricular fibroblasts were obtained from neonate rats using standard collagenase digestion methods, isolated from myocytes, and subsequently prepared for experimentation as described before (*25*). These preparations contained more than 95% cardiac fibroblasts as determined by morphological appearance and immunocytochemical staining and were previously demonstrated to express RXFP1 but not RXFP3 (*22*). The cells were maintained in DMEM and supplemented with 10% fetal calf serum and the antibiotics penicillin (50 units/mL) and streptomycin (50 μg/mL) (DMEM-FBS). These fibroblasts were used between passages 2 and 4 for all studies, while all described experiments were performed separately at least three to seven times in duplicate.

H3 Relaxin Dose−*Response Studies on Collagen Deposition*. To determine the optimal dose(s) at which H3 relaxin inhibited TGF-β1-stimulated collagen deposition, ventricular fibroblasts were plated at an equal density of 1×10^6 cells in six-well plate wells and supplemented with 1 mL (per well) of DMEM-FBS. The cells were then either untreated (controls) or treated for 72 h with TGF-β1 (3 ng/mL) alone or with TGF-β1 (3 ng/mL) plus increasing concentrations of H3 relaxin (10, 50, 100, 250, and 500 ng/mL) or with TGF-β1 (3 ng/mL) plus H2 relaxin (100 ng/mL; used as a positive control at a concentration that had previously been used to inhibit TGF-β1-stimulated collagen deposition (*22*)). The optimal dose at which H3 relaxin inhibited TGF-β1-stimulated collagen deposition was also tested alone over a 72 h culture period to determine if it affected basal collagen deposition. After 72 h, the deposited collagen into the cell layers (from each well) was isolated and hydrolyzed with 6 M hydrochloric acid for measurement of hydroxyproline content, as described previously (*26*, *27*). 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 (*28*)).

Western Blot Analysis of Smad2 Phosphorylation and α*-Smooth Muscle Actin* (*SMA*) *and MMP-13 Expression*. To determine if H3 relaxin had any effects on Smad2 phosphorylation (pSmad2; a key mediator of TGF-β1 signal transduction) and α-SMA expression (a marker of myofibroblast differentiation), which are both downregulated by H2 relaxin (*22*, *29*−*31*), and MMP-13 (collagenase-3, an enzyme involved in collagen degradation), which is upregulated by H2 relaxin (*32*, *33*), ventricular fibroblasts were plated at an equal density of 1×10^5 cells in 12-well plate wells and supplemented with 1 mL DMEM-FBS. The cells were then either untreated or treated for 72 h with TGF-β1 (3 ng/mL) alone or with TGF-β1 (3 ng/mL) plus H3 relaxin (100 ng/mL) or with TGF-β1 (3 ng/mL) plus H2 relaxin (100 ng/mL; positive control). After 72 h, the cell layers were subjected to Trizol reagent, and total protein was extracted from each sample (according to the manufacturer's instructions; Life Technologies, Gaithesburg, MD) and quantified by the Bio-Rad dye-binding protein assay (Bio-Rad Laboratories, Richmond, CA).

Equal protein extracts (in 1% sodium dodecyl sulfate; 10−15 μg of total protein/lane) were analyzed by electrophoresis under nonreducing conditions on 12.5% acrylamide gels, as described before (*22*). Western blot analysis was then performed with primary monoclonal antibodies to either pSmad2 (no. 3108; 1:1000 dilution; Cell Signaling Technology, Danvers, MA), α-SMA (M0851; 1:1000 dilution; Dako Corp., Carpinteria, CA), or MMP-13 (IM78T; 1:1000 dilution; Calbiochem, San Diego, CA) and either goat anti-rabbit (for pSmad2; 1:2500 dilution; Bio-Rad) or goat anti-mouse (for α-SMA and MMP-13; 1:2000 dilution; Bio-Rad) secondary antibodies as detailed previously (*22*, *30*, *33*). Membranes probed for pSmad2 were stripped and reprobed with a Smad2 polyclonal antibody (no. 3102; 1:750 dilution; Cell Signaling Technology) for determination of basal Smad2 levels and equal loading of the samples, while a monoclonal antibody to α-tubulin (05-829; 1:8000 dilution; Millipore Corp., Bedford, MA) was used to determine equal loading of samples for α-SMA and MMP-13. Densitometry of the pSmad, α-SMA, and MMP-13 bands was performed using a Bio-Rad GS710 calibrated imaging densitometer and Quantity-One software (Bio-Rad).

Gelatin Zymography of MMP-9 Expression and Activity. Of the main collagen degrading enzymes (MMP-2, -9, and -13) that have been shown to be upregulated by H2 relaxin in rodent models of disease (*5*, *22*, *29*, *31*−*33*), MMP-2 has previously been shown to be stimulated in a dose-dependent manner by H3 relaxin (*18*). Hence we decided to focus on the effects of H3 relaxin on MMP-9 and -13 in this study.

To assess the effects of H3 relaxin on the latent and active levels of MMP-9, ventricular fibroblasts were plated at an equal

density of 1×10^5 cells in 12-well plate wells and supplemented with 1 mL (per well) of DMEM-FBS. The cells were then either untreated (controls) or treated for 72 h with TGF-β1 (3 ng/mL) alone or with TGF-β1 (3 ng/mL) plus H3 relaxin (100 ng/mL) or with TGF-β1 (3 ng/mL) plus H2 relaxin (100 ng/mL; positive control), the final 24 h of treatment under serum-free conditions (as serum interferes with zymographic analysis of MMPs). 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 (*22*, *33*). Clear bands indicated gelatinolytic activity, while densitometry of the (latent and active) MMP-9 bands was performed as described above.

Reverse Zymography of Tissue Inhibitor of Metalloproteinase (TIMP) 1 and TIMP-2 Expression. To determine the effects of H3 relaxin on TIMP-1 (the natural inhibitor of MMP-9 and MMP-13) and TIMP-2 (the natural inhibitor of MMP-2) expression, equal aliquots of the same media samples (from untreated and TGF-β1 (3 ng/mL) ± H3 relaxin (100 ng/mL) or H2 relaxin (100 ng/mL) treated fibroblasts) that were used to identify changes in MMP-9 activity (by gelatin zymography) were also assessed by reversed zymography as described before (*34*). Densitometry of the TIMP-1 and TIMP-2 bands was performed as described above.

Assessment of the Synergistic Effects of H2 and H3 Relaxin on Collagen Deposition. To determine if H3 relaxin had any synergistic effects to the collagen inhibitory actions of H2 relaxin (via RXFP1 (*22*)), fibroblasts were plated at an equal density of 1×10^6 cells in six-well plate wells and supplemented with 1 mL of DMEM-FBS. The cells were then either untreated or treated for 72 h with TGF-β1 (3 ng/mL) alone or with TGF-β1 (3 ng/mL) plus H3 relaxin (50, 100 ng/mL), TGF-β1 (3 ng/mL) plus H2 relaxin (50, 100 ng/mL), or TGF-β1 (3 ng/mL) plus H3 relaxin (50 ng/mL) plus H2 relaxin (50 ng/mL). The collagen deposited into the cell layer of each sample over 72 h was then assessed by the hydroxyproline assay, as described above.

Assessment of the Antifibrotic Effects of H3 Relaxin in an Experimental Model of Fibrotic Cardiomyopathy in Vivo. The ability of H3 relaxin to reduce myocardial collagen accumulation *in vivo* was investigated in a well-established model of cardiac fibrosis, induced by cardiac-restricted transgenic overexpression of β2-AR, a model which had previously been used to evaluate the effects of H2 relaxin (*22*, *23*). The age at which cardiac fibrosis was already established in this model (4−5 months of age) was chosen for treatment studies, based on our previous observations (*22*, *23*). Transgenic animals ($n = 6-7$ per group) were subcutaneously implanted with osmotic minipumps (model 2002; DURECT Corp., Cupertino, CA) containing either a vehicle (20 mM sodium acetate buffer, pH 5.0) or synthetic H3 relaxin in vehicle at a concentration of 0.5 mg kg^{-1} day^{-1} (a concentration at which H2 relaxin has previously been used to assess its antifibrotic effects (*22*, *35*, *36*)). Age-matched wild-type littermate mice were used as controls. After 14 days, the left ventricle (LV) of each animal was isolated for hydroxyproline assessment of collagen content (*26*, *27*).

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

RESULTS

Synthesis and Characterization of H3 Relaxin. The human H3 relaxin A- and B-chains were synthesized separately by continuous flow Fmoc (fluoren-9-ylmethoxycarbonyl) solid-phase peptide synthesis (*18*). The two chains were combined using previously reported sequential and directed disulfide bond formation strategy (*18*, *37*, *38*). The final product was purified by preparative RP (reverse-phase) HPLC. The high purity of the peptide was confirmed by analytical RP-HPLC, and the identity was confirmed by MALDI-TOF mass spectrometry (calculated MH^+, 5499.52; found MH^+, 5498.51).

Effects of H3 Relaxin on TGF-β1-Stimulated Collagen Deposition. The optimal dose (10−500 ng/mL) at which H3 relaxin inhibited TGF-β1-stimulated collagen deposition over 72 h *in vitro* was determined by hydroxyproline analysis of fibroblast cell layers (Figure 1). TGF-β1 (3 ng/mL) alone significantly increased collagen deposition by 40−50% ($p < 0.01$ vs untreated cells) over 72 h in culture, consistent with its profibrotic actions and previously documented effects on rat cardiac fibroblasts (*22*). An inverse bell-shaped dose−response curve was observed with H3 relaxin treatment, where it did not have any marked effects on TGF-β1-stimulated collagen deposition at 10 and 50 ng/mL, significantly inhibited TGF-β1-stimulated collagen deposition at 100 and 250 ng/mL (back to levels measured in untreated control samples; both $p < 0.05$ vs TGF-β1 alone), but only induced a trend toward a decrease in TGF-β1-stimulated collagen deposition at 500 ng/mL (no significant difference vs TGF-β1 alone; Figure 1) over 72 h, with its optimal inhibitory actions being mediated at 100 ng/mL. At this dose, H3 relaxin was able to inhibit TGF-β1-stimulated collagen deposition to a similar extent as 100 ng/mL H2 relaxin (both $p < 0.05$ vs TGF-β1 alone, and back to levels measured in untreated control samples) over 72 h (Figure 1). However, H3 relaxin alone (100 ng/mL) did not affect basal collagen expression in the absence of TGF-β1 (Figure 1). Thus, 100 ng/mL H3 relaxin was used for subsequent experiments to assess its mechanisms of action (via RXFP1).

Effects of H3 Relaxin on pSmad2 and α-SMA Expression. To elucidate the mechanisms by which H3 relaxin may have affected TGF-β1-stimulated collagen production, its effects on pSmad2 and α-SMA expression were assessed by Western blotting. pSmad2 expression was barely detectable from untreated control fibroblasts but significantly upregulated by TGF-β1 (3 ng/mL) administration (which increased the pSmad2/Smad2 ratio by 120−130%; $p < 0.01$ vs untreated control group; Figure 2A) over 72 h of culture, consistent with pSmad2 being a positive regulator of TGF-β1 signaling (*39*). H3 relaxin (100 ng/mL) significantly inhibited TGF-β1-stimulated pSmad2 levels by ∼40% ($p < 0.05$ vs TGF-β1 alone) but not to the same extent as H2 relaxin (100 ng/mL), which inhibited TGF-β1-stimulated pSmad2 expression by ∼80% ($p < 0.01$ vs TGF-β1 alone; Figure 2A) over the same time period. pSmad2 expression was again barely detectable/undetectable in samples treated with H3 relaxin (100 ng/mL) alone, suggesting that H3 relaxin did not affect pSmad2 in the absence of TGF-β1 (Figure 2A).

TGF-β1 (3 ng/mL) administration to ventricular fibroblasts also significantly increased α-SMA expression by 70−80% over 72 h *in vitro* ($p < 0.01$ vs untreated control group; Figure 2B), consistent with its effects on collagen deposition (Figure 1) and pSmad2 (Figure 2A). H3 relaxin (100 ng/mL) was able to significantly inhibit TGF-β1-stimulated α-SMA expression to a similar extent as H2 relaxin (100 ng/mL) over 72 h in culture (both $p < 0.05$ vs TGF-β1 alone, and no difference to levels measured in untreated control samples; Figure 2B). However, H3 relaxin did not affect α-SMA expression in the absence of TGF-β1 (data not shown).

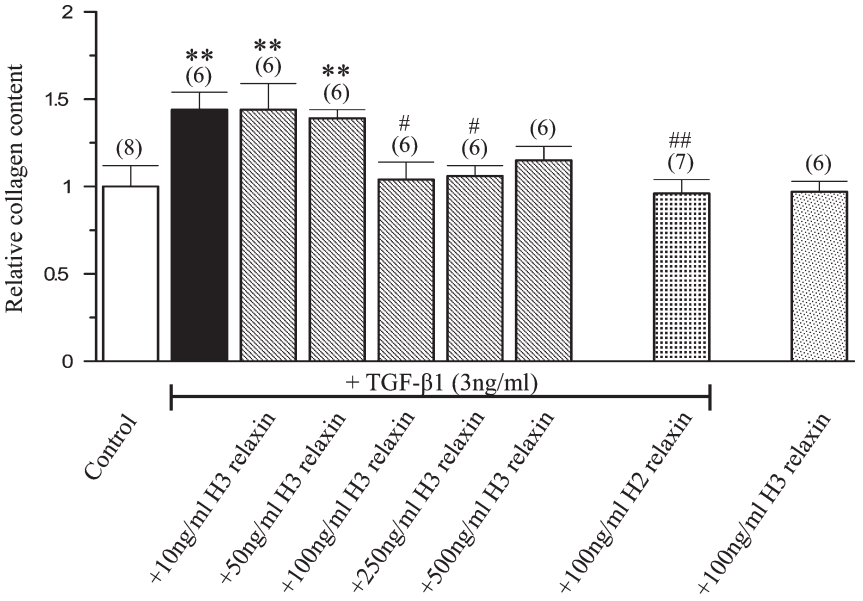

FIGURE 1: Dose−response studies. Shown is the relative mean ± SE collagen content from untreated (control) rat ventricular fibroblasts, TGF-β1-stimulated cells treated with increasing concentrations of H3 relaxin (0−500 ng/mL) or H2 relaxin (100 ng/mL), and H3 relaxin (100 ng/mL) alone treated fibroblasts after 72 h in culture. Numbers in parentheses represent the number of separate experiments conducted per group. **, $p < 0.01$ vs untreated/control group; #, $p < 0.05$; ##, $p < 0.01$ vs TGF-β1 alone.

Effects of H3 Relaxin on MMP and TIMP Expression. To elucidate the mechanisms by which H3 relaxin may have affected collagen breakdown, its effects on MMP-9 (by gelatin zymography), MMP-13 (by Western blotting), and TIMP-1 and TIMP-2 (by reverse zymography) were determined in the presence or absence of TGF-β1. TGF-β1 (3 ng/mL) administration to rat ventricular fibroblasts did not have any marked effects on MMP-13 (Figure 3A) or MMP-9 (Figure 3B) levels over 72 h compared to their respective levels measured from untreated control cultures. Likewise, H3 relaxin (100 ng/mL) did not have any significant effects on MMP-13 (Figure 3A) or MMP-9 (Figure 3B) in the presence of TGF-β1 compared to that measured in the untreated control and TGF-β1 alone treated groups. On the other hand, H2 relaxin (100 ng/mL) was able to significantly increase MMP-13 levels (by ~20%; $p < 0.05$ vs untreated control group; Figure 3A), without having any significant effect on MMP-9 expression (Figure 3B) in the presence of TGF-β1, over the same time period. Furthermore, H3 relaxin did not markedly affect MMP-13 (Figure 3A) or MMP-9 (Figure 3B) expression in the absence of TGF-β1.

TGF-β1 (3 ng/mL) did not markedly affect TIMP-1 or TIMP-2 expression over 72 h over basal levels measured from untreated control cultures, when added to rat ventricular fibroblasts (Figure 4). H3 relaxin (100 ng/mL) was able to significantly inhibit both TIMP-1 and TIMP-2 expression (by 50−55%) in the presence of TGF-β1 over 72 h (both $p < 0.05$ vs TGF-β1 alone and untreated control groups) and to a similar extent as that of H2 relaxin (100 ng/mL) in the presence of TGF-β1 (Figure 4). H3 relaxin (100 ng/mL), however, did not affect TIMP-1 or TIMP-2 expression in the absence of TGF-β1 (data not shown).

Effects of H3 Relaxin in Combination with H2 Relaxin on TGF-β1-Stimulated Collagen Deposition. To determine if H3 relaxin and H2 relaxin had any additive effects in inhibiting TGF-β1-stimulated collagen deposition, the effect of 50 ng/mL either peptide alone or in combination was assessed in the presence of TGF-β1 (3 ng/mL) (Figure 5). While neither H3 or H2 relaxin alone (at 50 ng/mL) significantly affected TGF-β1-stimulated collagen deposition over 72 h in culture, TGF-β1-stimulated collagen deposition was markedly inhibited in cells treated with H3 relaxin (50 ng/mL) in combination with H2 relaxin (50 ng/mL) over the same time period ($p < 0.05$ vs TGF-β1 alone, and back to levels measured in untreated control samples, as well as levels measured from adding 100 ng/mL of either peptide alone, in the presence of TGF-β1; Figure 5).

Effects of H3 Relaxin on Collagen Accumulation in a Rodent Model of Fibrotic Cardiomyopathy in Vivo. To extend the *in vitro* findings of inhibited TGF-β1-stimulated cardiac collagen deposition by H3 relaxin, its effects were also evaluated in 4−5-month-old β2-AR mice, which had previously been used to determine the antifibrotic effects of H2 relaxin (*22*, *23*). Cardiac fibrosis was established in 4−5-month-old β2-AR mice, as evidenced by a 70−75% increase in LV collagen concentration compared to that measured in age-matched wild-type animals ($p < 0.01$ vs wild-type mice; Figure 6). This increase in (β2-AR-transgenic expression-associated) aberrant LV collagen concentration was significantly reversed, however, in animals exposed to H3 relaxin administration (at a dose of 15 μg/day over a 14-day treatment period; by ~50%; $p < 0.05$ vs vehicle-treated β2-AR mice; Figure 6).

DISCUSSION

At the *in vitro* level, H3 relaxin was found to significantly inhibit TGF-β1 activity (at the level of pSmad2) and hence TGF-β1-stimulated myofibroblast differentiation, collagen deposition, and TIMP expression in a similar manner to that of H2 relaxin, via RXFP1. In fact, lower doses of H3 and H2 relaxin were found to additively lower TGF-β1-stimulated collagen deposition to levels induced by higher concentrations of either peptide alone, suggesting that H3 relaxin may augment the antifibrotic effects of physiological levels of H2 relaxin via RXFP1. At the *in vivo* level, the antifibrotic potential of H3 relaxin was further confirmed in a well-established mouse model of fibrotic cardiomyopathy, where it was found to reverse established cardiac fibrosis with similar

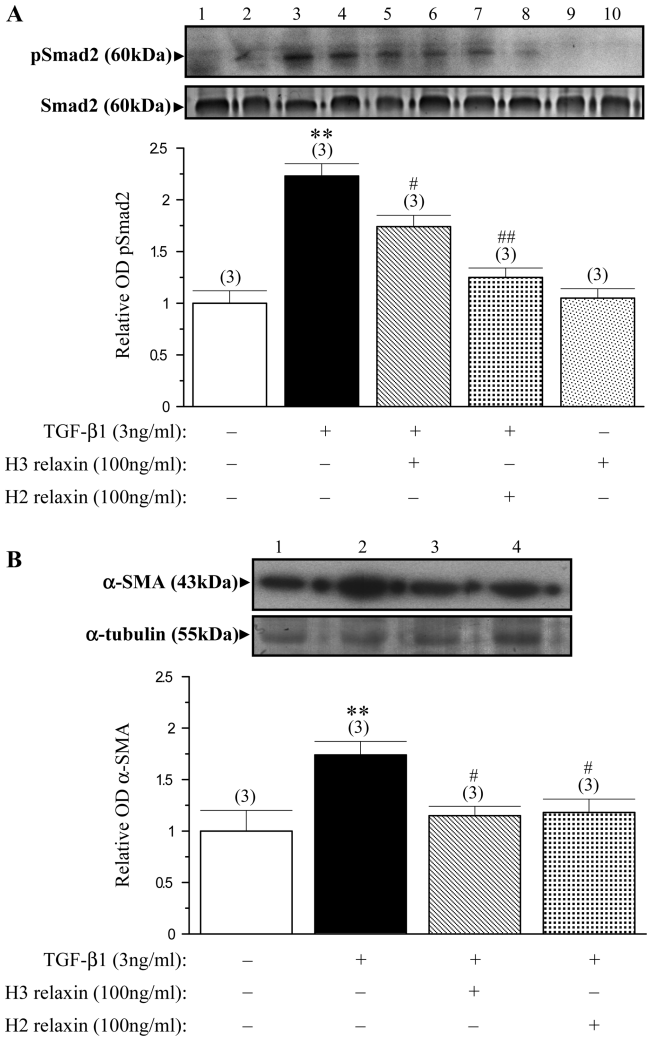

FIGURE 2: Effects of H3 relaxin on Smad2 phosphorylation (pSmad2) (A) and myofibroblast differentiation (α-SMA) (B). Shown are representative Western blots (from three separate experiments) of pSmad2 (A) from untreated/control ventricular fibroblasts (lanes 1 and 2) and TGF-β1 alone (lanes 3 and 4), TGF-β1 + H3 relaxin (lanes 5 and 6), TGF-β1 + H2 relaxin (lanes 7 and 8), or H3 relaxin alone (lanes 9−10) treated cells after 72 h in culture. Also shown are representative Western blots (from three separate experiments) of α-SMA (B) from untreated/control ventricular fibroblasts (lane 1) and TGF-β1-alone (lane 2), TGF-β1 + H3 relaxin (lane 3), or TGF-β1 + H2 relaxin (lane 4) treated cells after 72 h in culture. Unphosphorylated Smad2 (A) and α-tubulin (B) were used to demonstrate equivalent loading of the protein samples. The relative mean ± SE OD pSmad2 (corrected for Smad2) (A) and α-SMA (corrected for α-tubulin) (B) from the three separate experiments is additionally shown. **, $p < 0.01$ vs untreated/control group; #, $p < 0.05$; ##, $p < 0.01$ vs TGF-β1 alone.

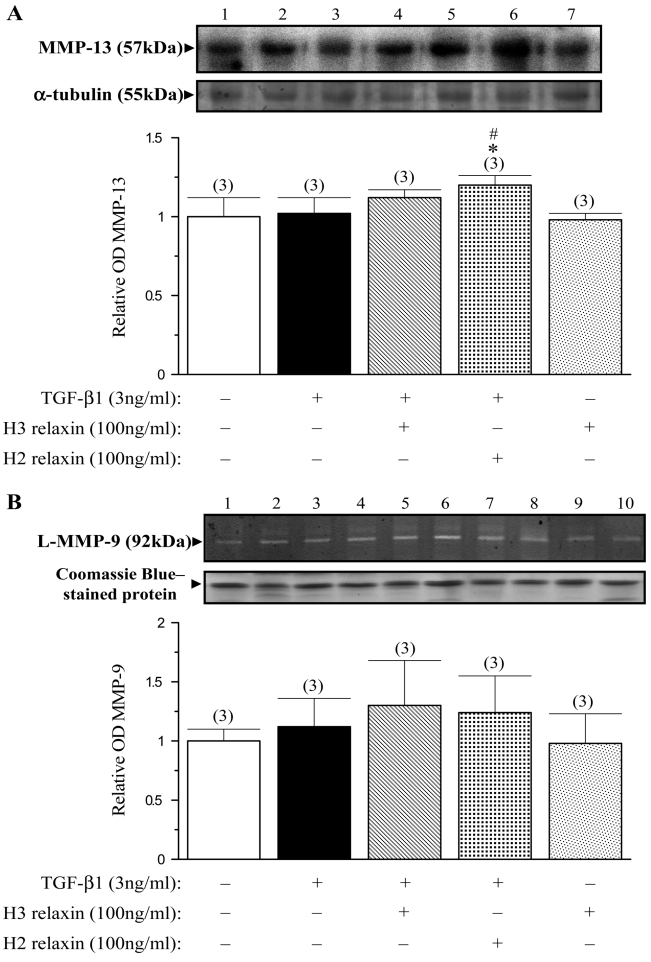

FIGURE 3: Effects of H3 relaxin on MMP-13 (A) and MMP-9 (B) levels. Shown are representative Western blots (from three separate experiments) of MMP-13 (A) from untreated/control ventricular fibroblasts (lane 1) and TGF-β1 alone (lane 2), TGF-β1 + H3 relaxin (lanes 3 and 4), TGF-β1 + H2 relaxin (lanes 5 and 6), or H3 relaxin alone (lane 7) treated cells after 72 h in culture. Also shown are representative gelatin zymographs (from three separate experiments) of MMP-9 (B) from untreated/control ventricular fibroblasts (lanes 1 and 2) and TGF-β1 alone (lanes 3 and 4), TGF-β1 + H3 relaxin (lanes 5 and 6), TGF-β1 + H2 relaxin (lanes 7 and 8), or H3 relaxin alone (lanes 9 and 10) treated cells after 72 h in culture. α-Tubulin (A) and a Coomassie Blue-stained protein (B) were used to demonstrate equivalent loading of the protein samples. The relative mean ± SE OD MMP-13 (corrected for α-tubulin) (A) and MMP-9 (corrected for protein) (B) from three separate experiments is additionally shown. *, $p < 0.05$ vs untreated/control group; #, $p < 0.05$ vs TGF-β1 alone.

efficacy to that of H2 relaxin (*22*) when continuously applied over a 2 week treatment period at an equivalent dose. Although the ventricular fibroblasts studied express RXFP1 (*22*) and RXFP2 (data not shown), but not RXFP3 mRNA (*18*), and the rat RXFP4 gene has been found to be a pseudogene which does not produce a functionally active receptor in this species(*40*), our previous findings that H3 relaxin only weakly binds to the rat RXFP2 receptor (*18*), while human INSL3 (the primary ligand for RXFP2) did not mimic the matrix remodeling actions of H2 relaxin (which acts via RXFP1) on rat renal myofibroblasts (*30*), strongly suggest that the reported actions of H3 relaxin on these cells were mediated via RXFP1 alone.

The inverse "bell-shaped" dose−response effects of H3 relaxin on TGF-β1-stimulated collagen deposition, which resulted in the highest concentration of H3 relaxin evaluated (500 ng/mL) not having the same inhibitory effects as lower doses (100−250 ng/mL), are consistent with previous findings for H2 relaxin (*35*, *41*−*43*) and other related peptides (*44*, *45*), which also produce biphasic dose−response curves, where higher concentrations of these peptides likewise do not produce the same maximum physiological responses. This is potentially explained from recent studies which have shown that RXFP1 undergoes dimerization and "negative cooperativity" (*45*, *46*) to accelerate the dissociation rate of relaxin peptides with ascending concentrations, leading to lower physiological responses of these peptides at higher concentrations. Additional studies though are required to fully understand this complex process and more importantly in

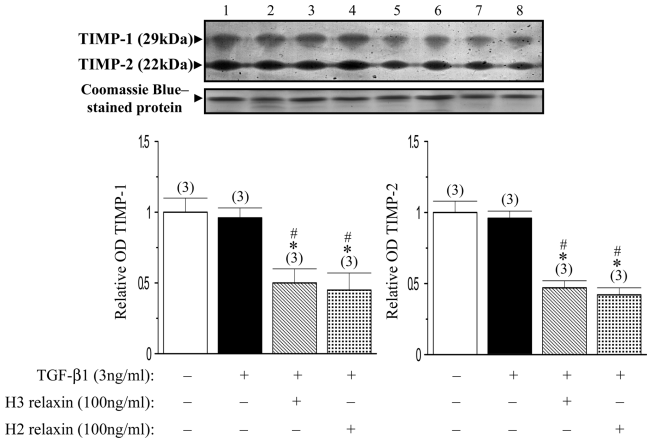

FIGURE 4: Effects of H3 relaxin on TIMP-1 and TIMP-2 levels. Shown is a representative reverse zymograph (from three separate experiments) of TIMP-1 and TIMP-2 from untreated/control ventricular fibroblasts (lanes 1 and 2) and TGF-β1 alone (lanes 3 and 4), TGF-β1 + H3 relaxin (lanes 5 and 6), or TGF-β1 + H2 relaxin (lanes 7 and 8) treated cells after 72 h in culture. A Coomassie Blue-stained protein was used to demonstrate equivalent loading of the protein samples. The relative mean ± SE OD TIMP-1 and TIMP-2 (corrected for protein) from three separate experiments is also shown. *, $p < 0.05$ vs untreated/control group; #, $p < 0.05$ vs TGF-β1 alone.

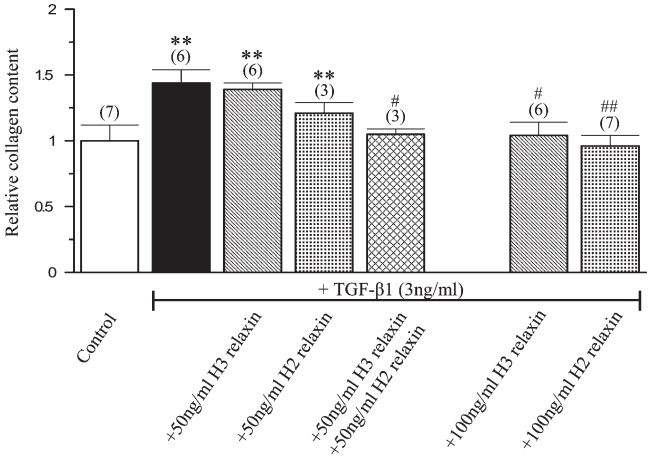

FIGURE 5: Synergistic effects of H3 and H2 relaxin on TGF-β1-stimulated collagen deposition. Shown is the relative mean ± SE relative collagen content from untreated (control) rat ventricular fibroblasts and TGF-β1-stimulated cells treated with H3 relaxin (50, 100 ng/mL) or H2 relaxin (50, 100 ng/mL), or H3 (50 ng/mL) and H2 relaxin (50 ng/mL), after 72 h in culture. Numbers in parentheses represent the number of separate experiments conducted per group. **, $p < 0.01$ vs untreated/control group; ‡, $p < 0.05$; ##, $p < 0.01$ vs TGF-β1 alone.

physiological cell systems (such as fibroblasts) that have low receptor numbers (*31*, *47*). As H3 relaxin has a lower affinity for RXFP1 (*18*) and reduced potency for accelerating its dissociation in comparison to H2 relaxin, it was thought that H3 relaxin was less likely to induce biphasic dose−response effects compared to H2 relaxin. Nevertheless, the findings of this study confirmed that H3 relaxin could significantly inhibit TGF-β1-stimulated but not basal collagen deposition, indicating that it exhibited rapidly acting but safe antifibrotic properties that mimicked the actions of H2 relaxin through binding to RXFP1.

Given that the affinity of H3 relaxin for the rat RXFP1 receptor is approximately 30−50-fold lower than that for H2 relaxin (*18*), it was not surprising that H3 relaxin was only able to

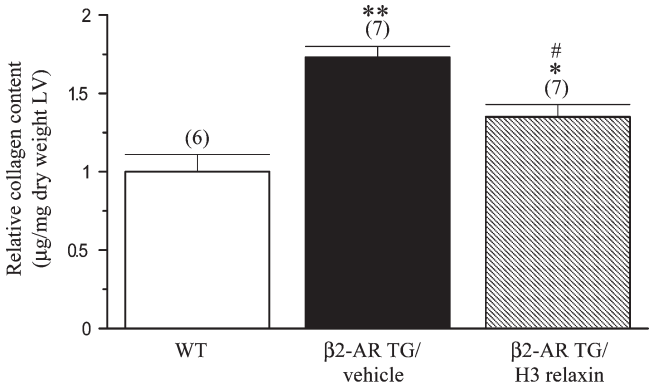

FIGURE 6: Effects of H3 relaxin in a mouse model of fibrotic cardiomyopathy *in vivo*. Shown is the relative collagen content (μg/mg dry weight LV tissue) from 4- to 5-month-old wild-type (WT) and age-matched littermate β2-AR transgenic (TG) mice treated with vehicle or synthetic H3 relaxin over a 14 day period. Numbers in parentheses represent the number of animals used per group. *, $p < 0.05$; **, $p < 0.01$ vs WT group; #, $p < 0.05$ vs β2-AR TG/vehicle group.

inhibit TGF-β1-mediated Smad2 phosphorylation by half the extent of H2 relaxin (~40% vs ~80%), while not being able to stimulate MMP-13 levels to the same extent as H2 relaxin. Interestingly though, H3 relaxin (at 100 ng/mL) was able to reduce TGF-β1-stimulated α-SMA expression (myofibroblast differentiation), collagen deposition, and TIMP-1 and TIMP-2 levels over 72 h in culture to a similar extent as the comparative dose of H2 relaxin. These findings may be attributed to the constant exposure of these peptides to RXFP1 receptors expressed on rat ventricular fibroblasts over the 72 h experimental period (with their activities stabilized and maintained by FBS), hence allowing them to exert inhibitory actions with similar efficacy over long-term culture periods *in vitro*. Additionally, although most GPCRs undergo desensitization in response to prolonged exposure to an agonist, it has recently been shown that prolonged RXFP1 signaling (to H2 relaxin (*48*), and presumably H3 relaxin) is likely attributed to its weak phosphorylation and poor internalization (in response to ligand binding) and the lack of its ability to recruit/traffic β-arrestins, which contributes to RXFP1 not being desensitized in the same manner as other GPCRs, allowing H2 and H3 relaxin to have comparable long-term effects via this receptor (on the downstream targets of TGF-β1/Smad2).

TGF-β1 is thought to have dual actions in response to cardiac injury, initially acting as an antiinflammatory mediator to stimulate ECM production during cardiac inflammation to promote wound healing (*49*), while its constant presence at the site of injury and its ability to auto-upregulate itself eventually results in an overproduction of matrix components (primarily collagen) and, hence, fibrosis (*50*). Hence, our findings that TGF-β1 promoted Smad2 phosphorylation, myofibroblast differentiation, and collagen deposition when administered to ventricular fibroblasts are consistent with its previously reported primary actions (*22*, *29*, *35*, *50*). On the other hand, our findings that TGF-β1 did not influence MMP-9, MMP-13, TIMP-1, or TIMP-2 levels in rat ventricular fibroblasts are consistent with its inability to alter MMP-9 and TIMP-2 activity in human lung fibroblasts (*51*) but differ from other studies which have shown that TGF-β1 suppresses collagen degradation by differentially regulating MMP activity (*52*) and upregulating TIMP-1 levels in human fibroblast cultures (*51*) and experimental models of heart disease (*53*). Additionally, although TGF-β1 was previously

found to promote MMP-2 expression and activity when administered to these rat ventricular fibroblasts (*18*, *22*) and other fibroblast culture models (*52*), it remains to be determined if this interaction is more consistent with its antiinflammatory and wound healing actions (*54*) or with its matrix-promoting effects. These combined findings suggest that the effects of TGF-β1 on MMPs and TIMPs (which remain controversial) are species, organ, and/or cell specific, or specific to the MMP and TIMP being studied, and are secondary to its effects on upregulating myofibroblast differentiation and collagen/matrix production.

Consistent with this, by suppressing TGF-β1 signaling (at the level of Smad2 phosphorylation), it is proposed that H3 and H2 relaxin mediate their antifibrotic actions by primarily inhibiting the predominant actions of TGF-β1 (on myofibroblast differentiation and collagen deposition), and to a lesser extent the secondary actions of TGF-β1 on collagen degradation, via regulation of the MMPs and TIMPs. This is the first study to demonstrate a H2 and H3 relaxin-mediated inhibition of Smad2 phosphorylation from nonrenal fibroblasts (*29*, *30*), implying that the regulation of pSmad2 is pivotal to the actions of these peptides in several organs outside the kidney. Despite not having any significant effects on MMP-9 activity (which is thought to play a role as an inflammatory mediator rather than as a regulator of fibrogenesis), our demonstration that H2 and H3 relaxin could promote MMP-2 levels (*18*) and downregulate TIMP-1 and TIMP-2 expression in TGF-β1-stimulated ventricular fibroblasts (while H2 relaxin could also increase MMP-13 levels) suggests that these peptides can additionally increase the MMP/TIMP balance that would favor a net promotion of matrix degradation in the presence of TGF-β1. The added finding that H3 relaxin did not affect basal collagen deposition (in the absence of TGF-β1) further suggests that H3 and H2 relaxins (*22*) do not have any direct effects on collagen per se.

At the *in vivo* level, continuous H3 relaxin infusion into a mouse model of fibrotic cardiomyopathy in which the etiology of cardiac fibrosis was independent of relaxin (i.e., due to cardiac-restricted overexpression of β2-adrenoreceptors) significantly reversed established collagen deposition by approximately 50%, again with similar efficacy to the 58% reduction in fibrosis that was seen with the same dose of H2 relaxin treatment of this model (*22*). These findings are consistent with the ability of H3 relaxin to ameliorate aberrant collagen deposition in an isoproterenol-induced model of cardiac toxicity in rats (*21*), when continuously applied over a 10 day period. The encouraging findings of these studies are that they demonstrate the rapid and specific reversal of cardiac fibrosis by short-term continuous treatment of H3 relaxin (over 10−14 days), which appear to be advantages to the modest effects of angiotensin converting enzyme inhibitors, which usually require longer treatment periods to demonstrate efficacy (*55*).

In conclusion, these studies have demonstrated that H3 relaxin can mimic the antifibrotic actions of H2 relaxin via RXFP1, most likely by interfering with TGF-β1 signaling and, hence, the influence of TGF-β1 on myofibroblast differentiation, collagen deposition, and collagen degradation (via regulation of the TIMPs). Although it has a lower affinity for RXFP1 compared to H2 relaxin, H3 relaxin was found to have similar efficacy to H2 relaxin when continuously exposed to fibroblast cultures *in vitro* and to an animal model of fibrotic cardiomyopathy *in vivo*. Furthermore, lower doses of H3 and H2 relaxin were found to additively inhibit TGF-β1-stimulated collagen deposition to the same extent as higher doses of each peptide alone, suggesting that H3 relaxin (acting through a similar mechanism via RXFP1) may provide a suitable adjunct therapy to H2 relaxin to maintain its efficacy at lower doses. Elucidating the signal transduction pathways that are activated by H3 relaxin to mediate its antifibrotic actions will be an important future step in determining the strengths and limitations of this peptide as a therapeutic agent and identifying novel therapeutic targets that may be used to enhance its antifibrotic potential. The findings of this study also demonstrated that H3 relaxin has important biological actions through other GPCRs outside its role(s) as a neuropeptide via RXFP3, where it was found to promote significant (but safe) matrix remodeling actions via RXFP1.

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