Evidence of AT$_1$R-AT$_2$R-RXFP1 Functional Crosstalk in Myofibroblasts and its Impact on the Therapeutic Targeting of Renal and Cardiac Fibrosis

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Running title: AT$_1$R-AT$_2$R-RXFP1 functional crosstalk

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ABSTRACT

Background Recombinant human H2 relaxin (serelaxin) has emerged as a potential agent to treat fibrosis, the pathological hallmark of chronic disease. As we now know that serelaxin requires the angiotensin II (Ang II) type 2 receptor (AT2R) to ameliorate renal fibrogenesis in vitro and in vivo, we sought to determine if its anti-fibrotic actions were affected by Ang II type 1 receptor (AT1R) modulation.

Methods We examined the signal transduction mechanisms of serelaxin when applied to primary rat renal and human cardiac myofibroblasts in vitro, and in three models of renal- or cardiomyopathy-induced fibrosis in vivo.

Results The anti-fibrotic signal transduction of serelaxin via its cognate receptor, relaxin family peptide receptor 1 (RXFP1), was abrogated by the AT1R blockers, irbesartan or candesartan in vitro and in vivo. Candesartan also ameliorated serelaxin’s anti-fibrotic actions in the left ventricle of mice with cardiomyopathy, indicating that the inhibitory effects of candesartan were not confined to the kidney. In a transfected cell system, we demonstrated that serelaxin did not directly bind to AT1Rs but that constitutive AT1R-RXFP1 interactions could form. To potentially explain these findings, we also demonstrated that all three receptors were expressed by renal and cardiac (myo)fibroblasts and that antagonists acting at each receptor directly/allosterically blocked the anti-fibrotic effects of either serelaxin or the AT2R agonist, Compound 21.

Conclusions These findings have significant implications for the concomitant use of RXFP1 or AT2R agonists with AT1R blockers and suggest that functional AT1R-AT2R-RXFP1 interactions on myofibroblasts may represent new targets for controlling fibrosis progression.

Keywords: Angiotensin receptors, fibrosis, kidney, heart, serelaxin, RXFP1
Fibrosis results from a failed wound healing response to tissue injury, and is a major contributor to organ dysfunction and failure.\textsuperscript{1} It is characterized by excessive extracellular matrix (ECM), and in particular, collagen accumulation and is a pathological feature of chronic kidney disease (CKD)\textsuperscript{2} and cardiovascular disease (CVD),\textsuperscript{3} irrespective of etiology. Numerous studies have shown that fibroblasts and other phenotypically similar mesenchymal cells are the predominant source of ECM, with \textit{de novo} expression of $\alpha$-smooth muscle actin ($\alpha$-SMA) in these cells indicative of their differentiation into activated myofibroblasts.\textsuperscript{4} Despite fibrosis representing a significant health burden, there are currently no effective strategies that directly ameliorate its progression.

The ovarian and cardiovascular hormone, relaxin, has several organ-protective actions that are mediated through its cognate G protein-coupled receptor, relaxin family peptide receptor 1 (RXFP1).\textsuperscript{5} The anti-fibrotic effects of human recombinant relaxin-2 (serelaxin; RLX) have been consistently demonstrated in experimental models of ageing,\textsuperscript{6,7} CKD and CVD.\textsuperscript{5,8-10} RLX directly inhibits angiotensin II (Ang II) and/or transforming growth factor (TGF)-$\beta$1-mediated fibroblast differentiation into myofibroblasts, prevents myofibroblast contraction\textsuperscript{11} and ECM/collagen synthesis by myofibroblasts.\textsuperscript{12,13} It also increases collagen-degrading matrix metalloproteinase (MMP) expression and activity.\textsuperscript{12-15} Additionally, RLX can inhibit inflammatory cell influx post-injury\textsuperscript{16-18} and influence macrophage polarization towards a M2 pro-repair macrophage phenotype.\textsuperscript{19}

Signal transduction studies have shown that activation of RXFP1 on renal and cardiac myofibroblasts by RLX directly increases cGMP\textsuperscript{20} or signals through an ERK1/2 phosphorylation and neuronal nitric oxide (NO) synthase (nNOS)-NO-sGC-cGMP-dependent pathway to inhibit TGF-$\beta$1 signal transduction at the level of intracellular Smad2 phosphorylation (phospho-Smad2).\textsuperscript{13,21-23} This in turn disrupts the pro-fibrotic actions of TGF-$\beta$1 on myofibroblast differentiation and aberrant ECM deposition. Likewise, RLX signals through this pathway in renal and dermal myofibroblasts to stimulate expression and activity of various collagen-degrading MMPs.\textsuperscript{24}

Intriguingly, Ang II type 2 receptor (AT$_2$R) antagonism or gene-deficiency abrogated the effects of RLX in renal myofibroblasts, suggesting that its anti-fibrotic actions required the AT$_2$R.\textsuperscript{25} These findings are consistent with the organ protection and inhibition of TGF-$\beta$1 signal transduction that is mediated by AT$_2$R activation.\textsuperscript{26-28} Furthermore, they may explain why RLX only affects injury-induced aberrant ECM/collagen deposition without affecting
basal matrix turnover, as the AT$_2$R is poorly expressed in healthy adult tissues and cells, but is dramatically up-regulated post-injury or after repeated stimulation and/or inflammation of the kidney and heart.$^{26, 29, 30}$

Along with RXFP1 and the AT$_2$R, the AT$_1$R is also expressed on (myo)fibroblasts$^{26, 31}$ and mediates the pro-fibrotic and vasoconstrictor actions of Ang II.$^{32}$ Accordingly, many AT$_1$R blockers (ARBs) are now in clinical use for their anti-hypertensive and to a lesser extent, anti-fibrotic actions.$^{33, 34}$ There is evidence that heteromers constitutively form between AT$_1$R and AT$_2$R, with reports suggesting that this impairs AT$_1$R internalization depending on the cell-type studied.$^{35}$ This may also explain how the AT$_2$R can inhibit the growth effects of the AT$_1$R.$^{36, 37}$ As heteromers can also form between RXFP1 and the AT$_2$R,$^{25}$ this raises the question as to whether functional interactions can occur between all three receptors on myofibroblasts, and whether this receptor crosstalk can be targeted to regulate fibrosis progression and/or the anti-fibrotic actions of RLX (acting at RXFP1) or an AT$_2$R agonist (acting at the AT$_2$R). In this study, we have addressed these important questions using primary myofibroblast cultures and murine models of fibrosis, together with selective AT$_1$R and AT$_2$R agonists and antagonists.
METHODS

Materials

Recombinant H2 relaxin was generously provided by Corthera Inc (San Carlos, CA, USA; a subsidiary of Novartis AG, Basel, Switzerland) and is bioactive in rats\textsuperscript{21, 22, 24, 25, 38-40} and mice\textsuperscript{19, 25, 39, 41, 42}. Irbesartan was kindly provided by Professor Clive May (Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria, Australia; candesartan cilexetil was obtained from AstraZeneca (Södertälje, Sweden); CGP42112 was obtained from GL Biochem (Shanghai) Ltd (Shanghai, China); Compound 21 was obtained from Vicore Pharma AB (Mölndal, Sweden); and PD123319 was obtained from Sigma-Aldrich (St Louis, MO, USA).

Animals

All animals used in this study were housed in a controlled environment and maintained on a fixed 12 hour light:12 hour dark schedule with free access to rodent lab chow (Barastock Stockfeeds, Pakenham, Victoria, Australia) and water. All experiments described below were approved by the Florey Institute of Neuroscience and Mental Health’s and Monash University’s Animal Ethics Committees, which adhere to the Australian Code of Practice for the Care and Use of Laboratory Animals for Scientific Purposes.

A rat and mouse model of UUO, which mimics the pathology of human progressive renal disease\textsuperscript{43, 44}, was used as an experimental model of primary tubulointerstitial fibrosis. In each case, a single ureter was ligated under general anesthesia, while the other kidney remained intact. Tissues were collected from male Sprague-Dawley rats (~250g in weight; obtained from the Animal Resource Centre, Perth, Western Australia, Australia) for the propagation of renal (myo)fibroblasts. Seven-eight week old male C57BL/6 mice (obtained from Monash University Animal Services, Clayton, Victoria, Australia) were also subjected to UUO, and either left untreated or treated with RLX (0.5mg/kg/day\textsuperscript{44}), in the absence or presence of candesartan cilexetil (2mg/kg/day; a dose used to demonstrate its therapeutic efficacy\textsuperscript{45, 46}) (n=5-6 mice per treatment group). RLX was delivered to sub-groups of mice via 7 day osmotic mini-pumps (model 1007D; Alzet®, Cupertino, CA, USA) from 2 days prior to injury until 5 days post-injury; while mice receiving candesartan cilexetil alone or in combination with RLX were administered the ARB via drinking water over the equivalent 7 day-treatment period.
Seven-eight week-old male 129sv mice (obtained from the Animal Resource Centre Perth, Western Australia, Australia) were separately subjected to a repeated ISO-induced model of cardiomyopathy and fibrosis\textsuperscript{42, 47}. Mice received daily subcutaneous injections of isoprenaline hydrochloride (25mg/kg; Sigma-Aldrich, St Louis, MO, USA) over 5 consecutive days, then left for a further 12 days for left ventricular fibrosis to develop. RLX (0.5mg/kg/day) was delivered to sub-groups of mice via osmotic mini-pumps (model 2002; Alzet\textregistered) from days 0-17; while animals treated with candesartan cilexetil (0.5mg/kg/day) alone or in combination with RLX received the ARB via drinking water over the 17 day-treatment period (n=7-8 mice per treatment group).

Eight-ten week old male FVB/N mice were subjected to a high salt (HS; 5% sodium chloride) diet over 8 weeks. From weeks 5-8, sub-groups of mice received RLX (0.5mg/kg/day; via osmotic mini-pumps; model 2004; Alzet\textregistered) alone, candesartan cilexetil (2mg/kg/day; via drinking water) alone or both combined (n=7-8/group). HS-fed mice that were left untreated over 8 weeks were included as injury-controls, while mice fed a normal salt (NS; 0.5% sodium chloride) diet were included as no-injury controls.

**Blood Pressure Measurements**

Systolic blood pressure (SBP) was measured in all ISO-injured and HS-fed mice, as well as their respective control groups, using tail cuff plethysmography\textsuperscript{48} (MC4000 Blood Pressure Analysis Systems; Hatteras Instruments Inc, NC, USA). In each case, SBP was measured at day 0 and day 17 of the ISO model; and weekly over the 8-week HS model. At least 15-20 measurements per time point were pooled to obtain a mean for each animal.

**Cell Culture**

Primary renal cortical myofibroblasts were propagated from the kidneys of male Sprague-Dawley rats after 3 days of UUO (RRMFs), and maintained as previously described\textsuperscript{21, 22, 24, 25}. The response of these cells to RLX is very similar to that seen in TGF-\(\beta\)\textsubscript{1}-stimulated human renal fibroblasts\textsuperscript{13}. Cultures were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10\% fetal bovine serum (FBS), 2.2\% HEPES, 1\% L-glutamine, penicillin (50U/ml) and streptomycin (50\(\mu\)g/ml) (DMEM-FBS) at 37\(^\circ\)C. All described experiments were
independently replicated at 3-7 separate times in duplicate or triplicate, with RRMFs used between passages 18-25.

Primary human fetal cardiac fibroblasts (from male fetuses and containing a mixture of atrial and ventricular fibroblasts) were obtained from ScienCell (Carlsbad, CA, USA) and maintained as described before. Cells were grown in Medium 199 containing 5% FBS, penicillin (100U/ml) and streptomycin (100µg/ml) plus fibroblast cell growth supplement-2 (FGS-2, ScienCell) (M199-FBS) and maintained at 37°C under 5% CO₂. In each case, these cells were stimulated with TGF-β1 (5ng/ml) to promote their differentiation into myofibroblasts (HCMFs); and all experiments on HCMFs were independently carried out at least 4 separate times in triplicate, with HCMFs used between passages 2-4.

**Evaluating the Effects of AT₁R Blockade on the Anti-Fibrotic Effects of RLX and C21 In Vitro**

To determine if a functional interaction existed between RLX and the AT₁R, primary RRMFs were seeded into 12-well plates at an equal density of 1-1.25x10⁵ cells per well, and immediately treated with RLX (100ng/ml; 16.8nM), in the absence or presence of the AT₁R antagonist, irbesartan (0.1-10µM) for 72h. The effects of irbesartan alone (0.1-10µM) were separately evaluated over the same time-period, to ensure that it did not affect the various endpoints measured in the absence of RLX. In all experiments, untreated RRMFs were used as controls.

The effects of AT₁R blockade on the anti-fibrotic effects of RLX (acting at RXFP1), an AT₂R agonist (Compound 21; C21), and the combined effects of both RLX and C21 were then evaluated. RRMFs were seeded into 12-well plates at an equal density as above, and immediately treated with RLX (16.8nM; a dose consistently used to demonstrate its anti-fibrotic effects in vivo, 12, 14, 15, 21, 22, 24, 25) or C21 (1µM; a dose used to demonstrate its therapeutic effects in vivo, 49, 50) in the absence or presence of candesartan (10µM); or RLX (16.8nM) and C21 (1µM) and candesartan (10µM) for 72h. Again, untreated RRMFs were used as controls.

After 72h, proteins were extracted from cell layers with Trizol reagent (Invitrogen, Carlsbad, CA, USA; according to the manufacturer’s instructions); while the media was collected and stored for analysis by gelatin zymography.
To determine the effects of AT\(_1\)R antagonism on the acute ERK1/2-promoting actions of RLX, RRMFs were seeded into 48-well plates at a density of 1-1.25x10\(^5\) cells per well and allowed to grow overnight at 37°C in DMEM-FBS. Cells were serum starved for 4-6 hours, then treated with either irbesartan (0.1-10µM) or candesartan (0.1-10µM) for 1h, and after that with RLX (16.8nM) for 5 minutes.

**Evaluating the effects of RXFP1 blockade on the anti-fibrotic effects of RLX and C21 *in vitro***

The effects of RXFP1 blockade on the anti-fibrotic effects of RLX (acting at RXFP1) or C21 (acting at the AT\(_2\)R) were also evaluated. RRMFs were seeded into 12-well plates at an equal density as above, and immediately treated with RLX (16.8nM) or C21 (1µM), in the absence or presence of an RXFP1 antagonist (1µM\(^{39,51}\)); or the RXFP1 antagonist (1µM) alone for 72h. Again, untreated RRMFs were used as controls. After 72h, proteins were extracted from cell layers and media isolated as described above.

**Evaluating the Effects of RXFP1 or AT\(_1\)R ± AT\(_2\)R blockade on the Acute Effects of RLX and C21 *In Vitro***

To determine the effects of RXFP1, AT\(_1\)R ± AT\(_2\)R antagonism on the acute cGMP-promoting actions of RLX and C21, primary human fetal cardiac fibroblasts (ScienCell, Carlsbad, CA, USA; containing a mixture of atrial and ventricular fibroblasts) were stimulated with TGF-β1 (5ng/ml) to stimulate the differentiation of these cells into myofibroblasts (HCMFs). HCMFs were seeded into 48-well plates at a density of 1-1.5x10\(^5\) cells per well and allowed to grow overnight at 37°C in M199-FBS. The following day, the cells were serum starved overnight, then treated with the RXFP1 antagonist (1µM) alone, candesartan (1µM) alone, the AT\(_2\)R antagonist, PD123319 (1µM) alone or candesartan (1µM)+PD123319 (1µM) for 30 minutes, then with either RLX (1nM) or C21 (1µM) for 40 minutes.

**Western Blotting**

Equal amounts of total protein (10-25µg; from mouse tissue extracts and RRMFs) from each sample were electrophoresed on 10.5% acrylamide gels, as described before\(^{21,25}\). Blots were
probed with primary polyclonal antibodies to either phosphorylated (phospho)-ERK1/2 (#9101 (Thr202/Tyr204); 1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), nNOS (#610308; 1:500 dilution; BD Biosciences, San Jose, CA, USA), phospho-nNOS (#PA1-032 (Ser1417); 1:1000 dilution; Pierce Biotechnology, Rockford, IL, USA), or TGF-β1 (#sc-146; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); or with primary monoclonal antibodies to phospho-Smad2 (#3108 (Ser465/467); 1:1000 dilution; Cell Signaling Technology), α-SMA (#M0851; 1:750 dilution; Dako Corporation, Carpinteria, CA, USA), MMP-13 (#ab75606; 1:500 dilution; Abcam, Cambridge, MA, USA), collagen I (either with an ab88147 monoclonal antibody (at 1:1000 dilution) that detected a single band corresponding to collagen I (Abcam); or with the ab34710 polyclonal antibody (at 1:1000 dilution) that detected the α1 and α2 chains of collagen I (Abcam)), or the AT1R (#sc-1173 (N-10); 1:1000 dilution; Santa Cruz Biotechnology); and the appropriate secondary antibodies via the use of Dako anti-rabbit or anti-mouse kits (Dako Corporation). Membranes probed with phospho-ERK1/2 and phospho-Smad2 were stripped and re-probed with total ERK1/2 (#4695 (137F5); 1:1000 dilution; Cell Signaling Technology) and Smad2/3 (#3102; 1:1000 dilution; Cell Signaling Technology), respectively. In each experiment, a single replicate membrane was probed for α-tubulin (#3873 (clone DM1A); 1:1000 dilution; Cell Signaling Technology) as a correction for semi-quantitative analysis of each other end-point measured. All blots were detected with the ECL detection kit and the appropriate bands quantified by densitometry using a GS710 Calibrated Imaging Densitometer and Quantity-One software (Bio-Rad Laboratories, Richmond, CA, USA). The optical density (OD) of each parameter measured was corrected for α-tubulin protein levels (or total ERK1/2 and Smad2/3 levels for phospho-ERK1/2 and phospho-Smad2, respectively) and expressed relative to the UUO-injured/untreated wild-type mouse group (in vivo studies) or the untreated control group (in vitro) studies, which was expressed as 1 in each case.

**Gelatin Zymography**

Equal amounts of mouse tissue extracts or cell culture media (5-10μg per sample) were evaluated by gelatin zymography, using 7.5% acrylamide gels containing 1mg/ml gelatin (Sigma-Aldrich, St Louis, MO, USA) as detailed previously24, 25. Gelatinolytic activity was indicated by clear bands and densitometry was used to assess changes in both the combined latent (L) and active (A) forms of either MMP-2 (gelatinase A) or MMP-9 (gelatinase B).
**Hydroxyproline Assay**

Equivalent tissue portions of kidney tissue (containing cortex and medulla) or left ventricular tissue (apical region) were lyophilized to dry weight, hydrolyzed in 6M hydrochloric acid and evaluated for hydroxyproline content as detailed before21, 25, 42. Hydroxyproline values were then converted to collagen content and collagen concentration (% collagen content/dry weight tissue52).

**Histology, Immunohistochemistry and Immunofluorescence**

Renal collagen IV and collagen I were identified from serial (5μm) paraffin-embedded kidney sections from each animal subjected to UUO-induced injury, using goat anti-collagen IV (#1340-01; 1:300 dilution; Southern Biotechnology, Birmingham, AL, USA) or rabbit anti-collagen I (#T40777R; 1:1000 dilution; Meridian Life Science, Memphis, TN, USA) primary antibodies; visualized; and morphometrically evaluated by point-counting25, 44. Equivalent areas of the renal cortex were evaluated for each collagen sub-type, with glomeruli and large vessels excluded from the analysis. Kidney tissues from NS and HS-fed mice were subjected to Masson’s trichrome staining (by Monash Histology Services, Clayton, Victoria, Australia) and analysed for changes in interstitial collagen deposition and glomerulosclerosis53.

Interstitial left ventricular collagen deposition was identified from paraffin-embedded (5μm) tissue sections that were stained with 0.1% picrosirius red (Polysciences Inc, Warrington, PA, USA). Images from each section were captured and analysed as described previously42; and data expressed as % collagen staining per total field area measured.

Immunofluorescence staining of renal phosphorylated-IκB levels (as a surrogate marker of NF-κB activity) was also measured from sections derived from NS and HS-fed mice, using a rabbit polyclonal primary antibody (#28595; 1:50 dilution; Cell Signaling Technology) and goat anti-rat IgG Alexa Fluor® 488 secondary antibody (#A11006; 1:500 dilution; Life Technologies, Camarillo, CA, USA).

All immunohistochemically- and immunofluorescently-stained slides, for targets other than collagen I and IV, were then scanned and captured using the Aperio AT Turbo scanner or Aperio FL scanner, respectively (Leica Biosystems, Nussloch, Germany); and stored as digital...
high resolution images on a local server associated with each instrument. Images were viewed and morphometrically analysed with the Aperio ImageScope v.12.1.0.5029 software (Leica Biosystems, Nussloch, Germany). In each case, images from 6-to-8 non-overlapping fields were captured (at the highest magnification available (x40)), analysed and data expressed as % staining per total field area.

**Competition-Binding Assays**

Whole-cell competition bindings assays (n=4 separate times) were conducted in human embryonic kidney (HEK)293 cells stably transfected with the AT_1R^54; where unlabeled RLX was compared against candesartan (AT_1R antagonist) and CGP42112 (AT_2R agonist), which were all prepared in DMEM containing 0.1% bovine serum albumin (BSA) and evaluated at concentrations ranging from 1pM to 1μM. In each separate experiment, each ligand concentration was assessed in triplicate, and the ability of each ligand to inhibit the specific binding of [^{125}I]Sar^1Ile^8Ang II was assessed. Nonlinear regression of the data was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

**BRET Assays**

BRET saturation assays were performed in HEK293 cells as described previously^{25} (n=3 separate times). Cells were transiently co-transfected with a constant amount of Rluc8-tagged AT_1R and increasing amounts of Venus-tagged RXFP1. The expression levels of Rluc8- and Venus-tagged constructs for each BRET experiment were detected by luminescence (LUMIstar; BMG Labtech, Mornington, Victoria, Australia) and fluorescence (Envision; Perkin-Elmer, Waltham, MA, USA) measurements, respectively. The actual Receptor-Venus/Receptor-Rluc8 expression ratios were then plotted.

**AlphaScreen Phospho-ERK1/2 and cGMP Accumulation Assays**

Receptor-mediated phospho-ERK1/2 levels from RRMFs was determined using the ERK1/2 SureFire kit (TGR Biosciences, Hindmarsh, South Australia, Australia); while receptor-mediated cGMP activity was determined from RRMFs and HCMFs using the HTRF cGMP Detection kit (CisBio BioAssays, Bedford, MA, USA) - both according to the manufacturer’s
instructions. In each case, the NO donor, 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO; 10µM) was used as a positive control for cGMP activity.

**Real-Time PCR**

1 µg of RNA from rat renal cortical myofibroblasts (passages 38-40) as well as untreated and Ang II (0.1µM)-treated neonatal rat cardiac ventricular fibroblasts (passages 2-3) was extracted (n=2 samples per cell type and treatment) using Trizol reagent (Invitrogen Pty Ltd, Mt Waverley, Victoria, Australia), and converted to cDNA (5x iSCRIPT supermix, BioRad Life Sciences, Gladesville, New South Wales, Australia). RXFP1, AT1aR and AT2R gene expression were analyzed with TaqMan gene expression assays (Rxfp1: Rn01495351_m1; Agtr1a: Rn02758772_s1; Agtr2: Rn00560677_s1) and were duplexed with the internal housekeeping gene, 18s rRNA (Hs99999901_s1; Applied Biosystems, Life Technologies, Mulgrave, Victoria, Australia) using an Applied Biosystems 7900HT Fast RT-PCR system (Applied Biosystems Australia). Calculations of relative expression were carried out using the comparative cycle of threshold fluorescence (ΔCT) method.

**Statistical Analysis**

All data were analyzed by one-way analysis of variance followed by the Newman-Keuls *post-hoc* test for multiple comparisons between groups using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). All Western blot, histology/immunohistochemistry and PCR data are expressed as the mean ± standard deviation of the mean (SDM) or relative mean ± SDM, with a value of p<0.05 considered as statistically significant.
RESULTS

The Anti-Fibrotic Actions of RLX in vitro are Blocked by the AT1R Antagonist Irbesartan

We firstly examined the effect of culturing rat renal myofibroblasts (RRMFs), propagated from animals with unilateral ureteric obstruction (UUO), with 100ng/ml (16.8nM) RLX for 72 hours (h). RLX significantly promoted p42/p44 mitogen-activated protein kinase phosphorylation (phospho-ERK1/2), nNOS expression and phosphorylation (phospho-nNOS) by ~80-120% (Figure 1, A); down-regulated TGF-β1, phospho-Smad2 and α-SMA expression (myofibroblast differentiation) levels by ~30-50% (Figure 1, B); and up-regulated MMP-9 and MMP-2 expression/activity as well as MMP-13 expression levels by ~80-130% (Figure 1, C). All these RLX-induced effects were abrogated by co-administration of irbesartan (at 0.1-10μM; Figures 1, A-C), while irbesartan alone did not affect on basal expression of these measures (Supplemental Figure 1).

The Anti-Fibrotic Actions of RLX in vivo are Blocked by the AT1R Antagonist Candesartan Cilexetil

To substantiate these in vitro findings (Figure 1), we examined the effects of 0.5mg/kg/day of RLX in vivo, a dose that produces circulating levels of ~20ng/ml after 5 days\textsuperscript{41} and consistent anti-fibrotic effects.\textsuperscript{12, 15, 21, 25, 38, 41} We compared the effects of RLX with the AT1R blocker, candesartan cilexetil (2mg/kg/day; that produces anti-hypertensive and anti-fibrotic effects),\textsuperscript{55} in male wild-type mice subjected to 5 days of UUO-induced tubulointerstitial renal fibrosis. Compared to UUO-injured mice, candesartan cilexetil-treated mice showed a trend towards preventing renal collagen concentration, but which was not significantly different from that measured in the untreated UUO group (Supplementary Figure 2); consistent with the slow-acting effects of ARBs.\textsuperscript{56, 57} In comparison, RLX-treated mice had increased renal phospho-ERK1/2 (p42-44 MAPK; by ~150%), nNOS expression and phosphorylation (both by 150-160%) (Figure 2, A) and latent MMP-13 levels (by ~130%) (Figure 2, B); but reduced renal TGF-β1 levels, phospho-Smad2, α-SMA expression (all by 55-60%) (Figure 2, B) as well as total collagen concentration (Supplementary Figure 2; Figure 2, C), collagen I and collagen IV immunostaining (all by 50-70%) (Figure 2, C) by 5 days post-injury. These anti-fibrotic and signal transduction effects of RLX were abolished by co-administration of candesartan cilexetil over the same time period (Figure 2).
To confirm that these unexpected findings were not confined to the kidney, we also evaluated the cardioprotective effects of RLX (0.5mg/kg/day) in a murine model of isoprenaline (ISO)-induced cardiomyopathy, in which RLX reduced cardiac fibrosis.\textsuperscript{39, 42} A lower dose of candesartan cilexetil (0.5mg/kg/day) was used to avoid any confounding anti-hypertensive effects. Repeated ISO administration markedly increased left ventricular (LV) collagen concentration and picrosirius red-stained interstitial collagen deposition by day 17 (Figure 3, A). RLX reduced both the ISO-induced increase in LV collagen concentration and interstitial collagen deposition by ~50-60% (Figure 3, A). Candesartan cilexetil alone did not demonstrate any anti-fibrotic efficacy at this concentration, but inhibited the anti-fibrotic effects of RLX when co-administered in the ISO model (Figure 3, A).

The anti-fibrotic effects of RLX were additionally evaluated in a high salt (HS)-induced model of renal fibrosis, a model that displays significantly increased total kidney collagen concentration, interstitial fibrosis and glomerulosclerosis (by 0.4-4-fold) after 8 weeks (Figure 3, B) in the absence of any marked hypertension (Supplementary Figure 3). Once again, RLX (0.5mg/kg/day) normalized HS-induced total kidney collagen concentration and significantly decreased interstitial renal fibrosis and glomerulosclerosis (Figure 3, B), when administered subcutaneously from weeks 5-8. In comparison, candesartan cilexetil (2mg/kg/day) alone did not demonstrate any anti-fibrotic efficacy when administered over the same period, but did lower blood pressure and demonstrate anti-inflammatory effects (Supplementary Figure 3), confirming its therapeutic activity at the dose and time-frame investigated. When co-administered with RLX, candesartan again abrogated the anti-fibrotic effects of RLX in the kidneys of HS-fed mice (Figure 3, B); confirming that it could antagonize the anti-fibrotic effects of RLX in more than one experimental setting/organ.

**Constitutive Heteromers Can Form Between RXFP1 and the AT\textsubscript{1}R but RLX Does Not Directly Bind to the AT\textsubscript{1}R**

To determine if RLX could directly bind to the AT\textsubscript{1}R, competition binding assays were performed in human embryonic kidney (HEK)293 cells over-expressing the AT\textsubscript{1}R (Figure 4, A). However, only candesartan (positive control) competed for AT\textsubscript{1}R binding (pIC\textsubscript{50}: 9.33 \pm 0.07), whereas neither RLX nor the AT\textsubscript{2}R agonist, CGP42112 (used as a negative control) demonstrated any binding to the AT\textsubscript{1}R.
Bioluminescence resonance energy transfer (BRET) saturation assays were next carried out in HEK293 cells to investigate AT$_1$R:RXFP1 heteromerization, and provided evidence that constitutive complex formation between these two receptors could occur, with saturation occurring at a ratio of 0.06 (Figure 4, B). On the other hand, no evidence of heteromerization between RXFP1 and the thyrotropin-releasing hormone receptor 1 (TRHR1) was observed in a negative control interaction.

As the ability of RLX to stimulate phospho-ERK1/2 in vitro and in vivo was prevented by co-administration of the AT$_2$R antagonist PD123319, we reasoned that several RXFP1-expressing cells may co-express the AT$_2$R. We therefore examined the effects of candesartan or irbesartan on phospho-ERK1/2 responses to acute RLX administration (5 min as opposed to vs 72h; Figure 1, A) in primary RRMFs, which were found to express rxfp1, AT$_1$aR and AT$_2$R gene expression (Figure 4, C). Phospho-ERK1/2 levels 5 minutes after RLX addition to RRMFs were significantly increased, an effect that was fully blocked by either candesartan or irbesartan (0.1-10μM) co-treatment (Figure 4, C); confirming an acute functional RXFP1:AT$_1$R interaction in RRMFs.

**Evidence for AT$_1$R-AT$_2$R-RXFP1 Crosstalk on Primary RRMFs**

Our collective findings strongly suggested that the anti-fibrotic actions of RLX (through RXFP1) could be abrogated by pharmacological blockade of either the AT$_1$R or AT$_2$R. We therefore, determined if functional crosstalk was possible between all three receptors. This was done by evaluating if the anti-fibrotic effects of RLX (16.8nM) or the AT$_2$R agonist, Compound 21 (C21; 1μM) were affected by co-administration of the AT$_1$R antagonist candesartan (10μM) (Figure 5, A) or the RXFP1 antagonist, B-R13/17K$_{51}$ (1μM) (Figure 5, B) in RRMFs. C21 or RLX significantly inhibited renal myofibroblast differentiation (α-SMA expression) and collagen-I deposition over 72h in culture, by ~25-35% of that measured in untreated cultures (all p<0.05 vs untreated group). Strikingly, the anti-fibrotic effects of either agonist in isolation or combined were fully antagonized by co-administration of candesartan (Figure 5, A); while the anti-fibrotic effects of either agonist alone were also blocked by co-administration of the RXFP1 antagonist (Figure 5, B).
Evidence for AT\textsubscript{1}R-AT\textsubscript{2}R-RXFP1 Crosstalk on Acute Second Messenger Signaling in Primary Human Myofibroblasts and RRMFs

To examine if functional interactions between RXFP1, AT\textsubscript{1}R and AT\textsubscript{2}R could also be detected more proximal to the receptors in an acute second messenger assay, we evaluated the effects of RLX (1nM) and C21 (1\mu M) on cGMP signaling, in the absence or presence of antagonists in human primary cardiac myofibroblasts (HCMFs; ScienCell, Carlsbad, CA, USA) and primary RRMFs (Figures 6, A and B, respectively). In these studies, the effects of RLX or C21 on cGMP accumulation were investigated, as cGMP has been linked to the effects of both RLX\textsuperscript{21-25} and C21.\textsuperscript{58, 59} RLX or C21 both significantly increased cGMP levels when administered to HCMFs and RRMFs (Figures 6, A and B, respectively) by 1.6-1.8-fold after 40 minutes (all p<0.01 vs no treatment). The cGMP-promoting effects of either RLX (Figure 6, A) or C21 (Figure 6, B) were significantly inhibited by co-administration of the RXFP1 antagonist (1\mu M) alone, candesartan (1\mu M) alone, PD123319 (1\mu M) alone or the combined effects of candesartan (1\mu M) and PD123319 (1\mu M) – once again indicating that the anti-fibrotic effects of an RXFP1 or AT\textsubscript{2}R agonist could be blocked by antagonists acting at each receptor.
DISCUSSION

This study investigated for the first time whether RLX required the presence of the AT1R to mediate its anti-fibrotic actions. As evidence has been presented previously for antagonism of AT1R activity being protective against renal\textsuperscript{60} and cardiac\textsuperscript{55} fibrosis, it was hypothesized that RLX would disrupt the pro-fibrotic actions of the Ang II-AT1R-TGF-β1 axis and that the anti-fibrotic effects of RLX would be enhanced by AT1R antagonists. However, we found that both acute RLX signaling (Figure 4, D and Figure 6) and its ability to down-regulate functional endpoints associated with fibrosis (Figures 1-3 and Figure 5) and up-regulate various collagen-degrading MMPs (Figures 1-2) were abolished by co-administration of AT1R antagonists (irbesartan, candesartan) in all \textit{in vitro} and \textit{in vivo} assays performed. While candesartan alone did not demonstrate any marked anti-fibrotic efficacy in the \textit{in vivo} models studied (Supplemental Figure 2 and Figure 3), potentially due to its slow-acting actions\textsuperscript{56, 57}, it consistently blocked the effects of RLX in primary rat and human myofibroblasts (Figure 2 and Figures 5-6) as well as in the animals models evaluated (Figures 2-3). Collectively, this suggested that functional crosstalk could exist between RXFP1 and the AT1R in myofibroblasts when both receptors were expressed at sufficient levels.

While HEK293 cells over-expressing AT1Rs were used to confirm that RLX does not directly compete for AT1R binding (Figure 4, A), BRET studies demonstrated that RXFP1 could form constitutive heteromers with the AT1R (Figure 4, B). Hence, the presence of AT1R-RXFP1 heteromers provides a potential explanation for the finding that AT1R antagonists, acting on AT1Rs on myofibroblasts\textsuperscript{26, 31}, can prevent the anti-fibrotic actions of RLX that are mediated through RXFP1\textsuperscript{21}. Further work though is required to validate whether these receptors interact via heteromerisation in myofibroblasts.

Our collective findings, however, showing that the anti-fibrotic effects of RLX could be blocked by either AT1R or AT2R antagonists,\textsuperscript{25} further suggests that interactions beyond RLX-RXFP1-AT1R or RLX-RXFP1-AT2R can occur. It is possible that crosstalk between RXFP1-AT1R and RXFP1-AT2R heteromers allows all three receptors to functionally interact. Such signaling complexes may enable antagonists acting either at the AT1R or AT2R to allosterically block the effects of RLX at RXFP1 (Figure 7). Additionally, it would suggest that RXFP1 and AT1R antagonists may block the actions of an AT2R agonist, and we were able to show that the acute signaling and downstream anti-fibrotic effects of C21 were blocked by either a RXFP1 or an AT1R antagonist (Figures 5 and 6). These results are strongly supportive of all
these events occurring, only if a functional crosstalk between all three receptors existed on myofibroblasts.

We are mindful that the expression of these receptors on myofibroblasts does not guarantee that they interact. However, it is becoming increasingly apparent that several class A GPCRs including AT$_1$R and AT$_2$R, and RXFP1 form heteromers or receptor complexes that are required to modulate function. RXFP1 is widely expressed in several organs including the kidney and heart, is moderately decreased following injury in the rodent and human heart, and is down-regulated by $\beta_1$-adrenoceptor stimulation but up-regulated by $\alpha_1$-adrenoceptor stimulation. AT$_1$Rs are also widely distributed throughout the heart and kidney in both healthy and diseased states, but are significantly increased in expression in rodents and humans undergoing injury or disease, particularly when the renin angiotensin system (RAS) is up-regulated. In comparison, AT$_2$ receptors are expressed at low levels in tissues and fibroblasts under physiologically quiescent states (at higher levels in females than males), but are dramatically up-regulated in pathological conditions affecting the heart and kidney. Therefore, AT$_1$R-AT$_2$R-RXFP1 complexes and/or functional interactions are most likely to be observed under pathological conditions when components of the RAS are up-regulated. These conditions that display increased Ang II and TGF-$\beta_1$ are precisely those where RLX and C21 are known to mediate their anti-fibrotic effects against increased Ang II and TGF-$\beta_1$, whereas these interactions are less likely to occur under physiological conditions.

Extending beyond the AT$_1$R-AT$_2$R or RXFP1-AT$_2$R interactions that have previously been reported, our study clearly demonstrates for the first time that the anti-fibrotic effects of agonists acting at RXFP1 (RLX) or the AT$_2$R (C21) are prevented by AT$_1$R blockade; hence, the crosstalk between more than two receptors can allosterically influence agonist activity at multiple receptors. This implies that the anti-fibrotic effects of RLX and/or AT$_2$R agonists may be compromised by ARBs (as demonstrated in Figures 1-6). Recent clinical trials evaluating the vasodilatory benefits of RLX in patients with acute heart failure in a phase III trial showed that it did improve dyspnea and end-stage mortality at 180 days post-treatment, but a more recent extended phase III trial failed to demonstrate beneficial effects over patients receiving a placebo (Novartis press release; March 22nd, 2017). The initial phase III trial was not sufficiently powered to determine if patients receiving ARBs or other medications had compromised benefits to receiving RLX, so it will be of significant interest to determine if the recent trial can shed more light on whether ARBs negatively impacted the organ-protective effects of RLX at a clinical level. Furthermore, as one study at least has shown that combining
the anti-fibrotic effects of the ACE inhibitor, enalapril, with RLX enhanced the anti-fibrotic efficacy of enalapril alone while maintaining the anti-fibrotic efficacy of RLX[42], it would be of interest to also determine if ACE inhibitors could be used as substitutes to ARBs and used in combination with RLX to offer wider cardioprotection in experimental and clinical settings.

Previous experimental studies combining the effects of ARBs and C21 in vivo[49,71,72] have not demonstrated any negation of the organ-protective effects of C21, unlike the data obtained in this study which demonstrated that ARBs consistently negated the anti-fibrotic effects of RLX in vivo and in vitro. However, combining C21 with losartan or candesartan cilexetil did not demonstrate any additive effects in reducing renal fibrosis[72] or atherosclerosis[49] in experimental rat or mouse models of diabetes, respectively. Hence, further work is required to explain these discrepant findings in more complex models, particularly to determine whether the lack of any additive effects of the combination therapy on fibrosis regression were due to the inhibitory effects of the ARBs studied on C21 function in myofibroblasts.

In conclusion, we provide evidence that myofibroblasts express RXFP1, AT1R and AT2R and that heteromers can form between RXFP1 and the AT1R; while heteromer formation between RXFP1 and the AT2R[25] as well as AT1R and AT2R[35,37] has previously been proposed. We have also demonstrated in pathophysiological-relevant human and rodent myofibroblast culture models and murine models of fibrosis in vivo that the anti-fibrotic effects of RLX can be abrogated either by AT1R or AT2R antagonists[25]; and that AT1R or RXFP1 blockade also antagonizes the myofibroblast differentiation- and collagen-inhibitory effects of an AT2R ligand acting alone or in combination with RLX (acting at RXFP1) in RRMFs. These findings support the idea that AT1R-AT2R-RXFP1 crosstalk can exist on matrix-producing myofibroblasts, which RLX and C21 can activate to mediate their anti-fibrotic effects but which in turn, can be allosterically regulated by antagonists acting at the other receptors of this receptor interaction (Figure 7). Moreover, they suggest that this receptor crosstalk may represent a previously unrecognized target for the regulation and treatment of organ fibrosis. Clearly, future work is required to determine if this receptor interaction impacts on fibrosis progression in other organs where all three receptors are expressed; in non-myofibroblast cells that may impact on the vasodilatory and angiogenic actions of RXFP1 and AT2R agonists; and in conditions such as pregnancy[73,74] or preeclampsia[75,76], where an interaction between these receptors may influence relevant outcomes.
Acknowledgements

We are grateful to Drs Andreas Loening and Sanjiv Gambhir (Stanford University, Palo Alto, CA), and Dr Atsushi Miyawaki (RIKEN Brain Science Institute, Wakocity, Japan) for providing cDNA constructs (for the BRET assays); Professor Walter Thomas (University of Queensland) for providing the HEK293 cells stably transfected with AT1Rs; and Associate Professor Julie McMullen (Baker IDI Heart and Diabetes Institute) for providing RNA from unstimulated and Ang II-stimulated neonatal rat ventricular fibroblasts. This study was supported by a University of Melbourne Fee Remission Scholarship to B. S. M. Chow; Australian Postgraduate Scholarships to M. Shen, Y. Wang and G. Barsha; Monash University International Scholarships to C. Wang; National Health and Medical Research Council of Australia (NHMRC) Project Grants GNT0436713, GNT0454375, GNT0628634, GNT1045848 and GNT1101552; a NHMRC Program Grant GNT1055134 to R. J. Summers; NHMRC Senior Research Fellowships to R. A. D. Bathgate (GNT1042650), K. M. Denton (GNT1041844) and C. S Samuel (GNT1041766); and an NHMRC RD Wright Fellowship to K. D. G. Pfleger (GNT1085842). Research conducted at the Florey Institute of Neuroscience and Mental Health was additionally supported by the Victorian Government’s Operational Infrastructure Support Program.

Conflict of Interest Statement

K. D. G. Pfleger receives funding from, and is a shareholder of, Dimerix Limited, of which he is Chief Scientific Advisor.

Supplemental Material

Table of Contents:

Supplemental Figure 1: Irbesartan does not affect the signal transduction end-points associated with the anti-fibrotic actions of RLX, in the absence of RLX.

Supplemental Figure 2: Candesartan alone did not reduce renal fibrosis post-UUO at the time-point studied.
Supplemental Figure 3: Candesartan alone reduced systolic blood pressure and renal inflammation post-HS-induced nephropathy.
Figure Legends

Figure 1. The anti-fibrotic actions of RLX were blocked by the AT₁R antagonist irbesartan in vitro. (A) Representative Western blots of renal phosphorylated (phospho)-p44 and p42 MAPK (phospho-ERK1/2), total p44 and p42 MAPK (ERK1/2), neuronal nitric oxide synthase (nNOS), phospho-nNOS, transforming growth factor-β1 (TGF-β1), phospho-Smad2, total Smad2, α-smooth muscle actin (SMA); representative gelatin zymographs of latent (L) and active (A) matrix metalloproteinase (MMP)-9 and MMP-2 levels and representative Western blots of L-MMP-13 and α-tubulin expression from untreated (control) rat renal myofibroblasts and cells treated with RLX (16.8 nM) alone, or in the presence of irbesartan (0.1, 1, 10 μM) after 72 hours in culture. The total p44 and p42 MAPK (ERK1/2), unphosphorylated Smad2, and α-tubulin blots (A) were included to demonstrate the quality and equivalent loading of protein samples. (B) Also shown are the relative mean ± SEM optical density (OD) levels of phospho-ERK1/2 (corrected for total ERK1/2 levels); nNOS, and phospho-nNOS (both corrected for α-tubulin levels); TGF-β1, α-SMA (both corrected for α-tubulin levels), and phospho-Smad2 (corrected for total Smad2 levels); and MMP-9, MMP-2, and MMP-13 (corrected for α-tubulin levels) from each of the groups studied, as determined by densitometry scanning (from n=3-4 separate experiments conducted in duplicate), to that of the untreated group, which was expressed as 1 in each case. *p<0.01 vs untreated cells; #p<0.01 vs RLX alone-treated cells.

Figure 2. The anti-fibrotic actions of RLX in the injured kidney were blocked by the AT₁R antagonist candesartan in vivo. (A) Representative Western blots of renal phosphorylated (phospho)-p44 and p42 MAPK (phospho-ERK1/2), total p44 and p42 MAPK (ERK1/2), nNOS, phospho-nNOS, transforming growth factor-β1 (TGF-β1), phospho-Smad2, total Smad2, α-SMA, L-MMP-13 and α-tubulin. (B) Representative images of immunohistochemically-stained collagen I and collagen IV as well as total kidney collagen concentration in UUO-injured male C57B6J wild-type mice that were untreated for 5 days or treated with RLX (0.5mg/kg/day) alone, or RLX (0.5mg/kg/day) and candesartan cilexetil (2mg/kg/day) from 2 days prior to injury until 5 days post-injury. Scale bar = 100 μm. Total p44 and p42 MAPK (ERK1/2), unphosphorylated Smad2 and α-tubulin blots (A) were included to demonstrate the quality and equivalent loading of protein samples. Also shown (B) are the mean ± SEM total kidney collagen concentration (expressed as a % of the dry weight tissue) or renal collagen I
and collagen IV staining (expressed as a % of the fractional area stained, respectively) (from n=5-6 animals per treatment group). *p<0.01 vs UUO-injured/untreated group; #p<0.01 vs UUO-injured/RLX-treated group.

**Figure 3.** The anti-fibrotic actions of RLX in the heart of ISO-injured mice and kidneys of HS-fed mice were blocked by the AT1R antagonist candesartan *in vivo*. (A) Shown is the mean ± SEM total left ventricular collagen concentration; representative picrosirius red-stained images of left ventricular interstitial collagen deposition (scale bar = 100µm); and the mean ± SEM picrosirius red-stained left ventricular interstitial collagen deposition (as a % of the fractional area) from uninjured male 129sv mice, ISO (25mg/kg)-injured mice (after 17 days of injury) and ISO-injured mice treated with candesartan cilexetil (0.5mg/kg/day) alone, RLX (0.5mg/kg/day) alone or RLX (0.5mg/kg/day) and candesartan (0.5mg/kg/day) throughout the 17 days of ISO-induced injury (from n=7-8 animals per treatment group). (B) Also shown are the mean ± SEM total renal collagen concentration; representative Masson’s trichrome-stained images of renal interstitial collagen deposition (scale bar = 100µm); and the mean ± SEM interstitial renal collagen staining per field (expressed as a %) as well as the mean ± SEM glomerulosclerosis score (as assessed by morphometry of Masson’s trichrome stained images); from male FVB/N mice fed a high salt-(HS; 5% sodium chloride)-diet for 8 weeks and treated from weeks 5-8 with candesartan cilexetil (2mg/kg/day), RLX (0.5mg/kg/day) or both combined (n=7-8 animals per treatment group). *p<0.01 vs uninjured control groups, respectively; *p<0.01 vs ISO- or HS-injured/untreated group, respectively; #p<0.01 vs ISO- or HS-injured+candesartan-treated group, respectively; +p<0.01 vs ISO- or HS-injured+RLX-treated group, respectively.

**Figure 4.** RLX does not directly interact with the AT1R despite evidence for constitutive AT1R-RXFP1 heteromer formation. (A) Competition-binding assays in HEK293 cells over-expressing the AT1R demonstrated that RLX and an AT2R agonist (CGP42112) did not directly bind to the AT1R, whereas an AT1R antagonist (candesartan) did. (B) BRET saturation curves generated from transiently co-transfected HEK293 cells with a constant amount of Rluc8-tagged AT1R and increasing amounts of Venus-tagged RXFP1 are consistent with heteromers forming between RXFP1 and the AT1R (with saturation demonstrated with a maximum BRET ratio of 0.06), whereas no heteromerization appeared to occur between RXFP1 and the thyrotropin-releasing hormone receptor 1 (TRHR1), which was used as a negative control. (C)
(Myo)fibroblasts express RXFP1, AT<sub>1</sub>R and AT<sub>2</sub>R mRNA levels. Shown in the delta CT cycle at which RXFP1, AT<sub>1</sub>R and AT<sub>2</sub>R mRNA expression was detected, relative to the internal housekeeping gene 18s rRNA, by real-time PCR analysis of untreated and Ang II (0.1μM)-treated neonatal rat cardiac ventricular (myo)fibroblasts compared to rat renal myofibroblasts. Data were obtained from n=2 samples per group. NS denotes not significantly different compared to values from the untreated RCF group; †p<0.05 vs values from the RCF group; §p<0.05 vs values from the AngII-RCMF group. (D) The ability of RLX to significantly increase phospho-ERK1/2 levels (after 5 minutes) in rat renal myofibroblasts was abrogated by either candesartan or irbesartan (at 0.1⁻10μM) co-administration, indirectly confirming that several RXFP1-expressing cells were also co-expressing AT<sub>1</sub>R. All data are presented as the mean ± SEM relative to the untreated control group (which was expressed as 1); and obtained from n=3 separate experiments. *p<0.01 vs untreated cells; ††p<0.01 vs RLX alone-treated cells.

Figure 5. Evidence for AT<sub>1</sub>R-AT<sub>2</sub>R-RXFP1 interactions on RRMFs. (A) Representative Western blots of α-SMA and collagen I from untreated RRMFs and cells treated with C21 (AT<sub>2</sub>R agonist; 1μM) or RLX (16.8nM) alone, C21 (1μM) + candesartan (10μM), RLX (16.8nM) + candesartan (10μM) or C21 (1μM) + RLX (16.8nM) + candesartan (10μM) after 72 hours. (B) Representative Western blots of α-SMA and collagen I from untreated RRMFs and cells treated with C21 (1μM) or RLX (16.8nM) alone, an RXFP1 antagonist (antag; 1μM) alone, C21 (1μM) + RXFP1 antagonist (1μM) or RLX (16.8nM) + RXFP1 antagonist (1μM) after 72 hours. (A-B) In each case, α-tubulin staining was included to demonstrate the quality and equivalent loading of protein samples. Also shown are the relative mean ± SEM optical density (OD) levels of α-SMA and collagen I (both corrected for α-tubulin loading) from each of the treatment groups analysed (from n=4 separate experiments conducted in duplicate). *p<0.05 vs untreated cells; †p<0.01 vs C21 alone; ††p<0.01 vs RLX-alone.

Figure 6. Evidence for acute AT<sub>1</sub>R-AT<sub>2</sub>R-RXFP1 interactions on HCMFs and RRMFs. (A) cGMP accumulation was significantly increased by RLX (1nM) treatment of HCMFs or RRMFs after 40 minutes (n=4-7 experiments/cell type). (b) Likewise, cGMP accumulation was similarly increased by C21 (1μM) treatment of HMMFs or RRMFs after 40 minutes (n=4 experiments/cell type). (A-B) In each case, the cGMP-promoting effects of RLX or C21 in HCMFs and RRMFs were significantly inhibited by pre-treatment with an RXFP1 antagonist.
(1µM) alone, candesartan (1µM) alone, PD123319 (1µM) alone or candesartan (1µM)+PD123319 (1µM). Each antagonist alone, however, did not significantly affect basal cGMP measurements. *p<0.01 vs untreated cells; *p<0.05 vs RLX alone, †p<0.05 vs C21 alone.

**Figure 7.** A schematic illustration of how RLX potentially mediates its anti-fibrotic effects through AT₁R-AT₂R-RXFP1 complexes, which in turn, can be regulated by either AT₁R or AT₂R antagonists. (A) This study provides evidence for AT₁R-RXFP1 heteromers forming constitutively, while previous studies have indicated that AT₂R-RXFP1²⁵ and AT₁R-AT₂R³⁵,³⁷ heteromers can also constitutively form. (B) Based on our findings, we propose that crosstalk between RXFP1-AT₁R and RXFP1-AT₂R heteromers may be taking place on myofibroblasts. (C) This mechanism would allow antagonists acting at either the AT₁R or AT₂R to allosterically abrogate the anti-fibrotic effects of RLX that are mediated through these receptor functional interactions, respectively. Likewise, although not shown, this receptor interaction would allow AT₁R or RXFP1 antagonists to allosterically abrogate the anti-fibrotic effects of AT₂R agonists.
References


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Figure 1

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- Smad2 (60kDa)
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- L-MMP-13 (57kDa)
- α-tubulin (55kDa)

C

Collagen I

Collagen IV

- Total collagen
- Collagen I
- Collagen IV
Figure 3

A

Total collagen

Interstitial collagen

B

Total collagen

Interstitial collagen

Glomerulosclerosis
**Figure 4**

**A. AT₁R Competition Binding**

![Graph showing AT₁R competition binding with various ligands and concentrations.]

**B. RXFP1 and AT₁R constitute heteromers (BRET saturation curves)**

![Graph showing BRET ratio (minus donor-only control) versus fluorescence/luminescence for RXFP1 & AT₁R and RXFP1 & TRHR1 (neg. cont.).]

**C. Phospho-ERK1/2 (fold over basal)**

![Bar graphs showing phospho-ERK1/2 levels in Rat renal myofibroblasts with different treatments.]

**D. Phospho-ERK1/2 (Rat renal myofibroblasts)**

![Graph showing phospho-ERK1/2 levels with different treatments and concentrations.]

*RLX (16.8nM) - Irbesartan (µM): 0, 0.1, 1, 10 and Candesartan (µM): 0, 0.1, 1, 10.

*Statistical symbols:* *p < 0.05, #p < 0.01, **p < 0.001, NS = not significant.
Figure 5

A

Rat renal myofibroblasts

<table>
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<tr>
<th>Control (CTL)</th>
<th>C21</th>
<th>RLX</th>
<th>C21 + RLX + Antag</th>
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<tbody>
<tr>
<td>α-SMA (43kDa)</td>
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<td>Collagen-I (75kDa)</td>
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<tr>
<td>α-tubulin (50kDa)</td>
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B

<table>
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<tr>
<th>Control (CTL)</th>
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<th>RLX</th>
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<td>α-tubulin (50kDa)</td>
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</table>
Figure 7

A

B

C

Anti-fibrotic effects

Anti-fibrotic effects

Antagonists
Evidence of AT₁R-AT₂R-RXFP1 Functional Crosstalk in Myofibroblasts and its Impact on the Therapeutic Targeting of Renal and Cardiac Fibrosis

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Running title: AT₁R-AT₂R-RXFP1 functional crosstalk
SUPPLEMENTAL MATERIAL

Table of Contents:

Supplemental Figure 1: Irbesartan does not affect the signal transduction end-points associated with the anti-fibrotic actions of RLX, in the absence of RLX.

Supplemental Figure 2: Candesartan alone did not reduce renal fibrosis post-UUO at the time-point studied.

Supplemental Figure 3: Candesartan alone reduced systolic blood pressure and renal inflammation post-HS-induced nephropathy.
Supplemental Figure 1. Irbesartan does not affect the signal transduction end-points associated with the anti-fibrotic actions of RLX, in the absence of RLX. (A) Representative Western blots of renal phosphorylated (phospho)-p44 and p42 MAPK (phospho-ERK1/2), total p44 and p42
MAPK (ERK1/2), nNOS, phospho-nNOS, and α-tubulin; (B) TGF-β1, phospho-Smad2, total Smad2, α-SMA and α-tubulin; and (C) representative gelatin zymographs of latent (L) and active (A) MMP-9 and MMP-2 levels and representative Western blots of L-MMP-13 and α-tubulin expression from untreated (control) rat renal myofibroblasts and cells treated with irbesartan (0.1, 1, 10nM) after 72 hours in culture. The total (A) p44 and p42 MAPK (ERK1/2), (B) unphosphorylated Smad2, and (A-C) α-tubulin blots were included to demonstrate the quality and equivalent loading of protein samples. Also shown are the relative mean ± SEM optical density (OD) levels of (A) phospho-ERK1/2 (corrected for total ERK1/2 levels), nNOS, and phospho-nNOS (both corrected for α-tubulin levels); (B) TGF-β1, α-SMA (both corrected for α-tubulin levels), and phospho-Smad2 (corrected for total Smad2 levels); and (C) MMP-9, MMP-2, and MMP-13 (corrected for α-tubulin levels) from each of the groups studied, as determined by densitometry scanning (from n=3-4 separate experiments conducted in duplicate), to that of the untreated group, which was expressed as 1 in each case. NS denotes not significantly different compared to values from the untreated control group.
Supplemental Figure 2. Candesartan alone did not reduce renal fibrosis post-UUO at the time-point studied. Total renal collagen concentration (as determined from hydroxyproline analysis) was evaluated 5 days post-UUO in sub-groups of untreated mice vs those treated with candesartan (2mg/kg/day; via drinking water) alone, RLX (0.5mg/kg/day; via osmotic mini-pumps) or both combined; with all treatments administered from 2 days prior to UUO until 5 days post-UUO (from n=6-7 animals per treatment group). Candesartan alone only induced a trend towards preventing UUO-induced renal collagen concentration, potentially due it its slow-acting effects in this model, but was able to significantly abrogate the collagen-inhibitory effects of RLX. *p<0.05 vs UUO alone; #p<0.05 vs UUO+RLX group.
Supplemental Figure 3. Candesartan alone reduced systolic blood pressure and renal inflammation post-HS-induced nephropathy. (A) Candesartan (2mg/kg/day; via drinking water) administration to high salt (HS; 5% NaCl)-fed mice, from weeks 5-8 of the 8 week model, significantly lowered systolic blood pressure (after 4 weeks of administration; at week 8). (B) Candesartan also significantly lowered the HS-induced increase in phosphorylated-IκB immunostaining (which was used as a surrogate marker of NF-κB activity) after 4 weeks of administration (at week 8) (n=6-8 animals per treatment group); indicative of its therapeutic efficacy in this model. Scale bar = 50μm.*p<0.05 vs normal salt (NS)-fed group; #p<0.05 vs HS-fed group.