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Relaxin requires the angiotensin II type 2 receptor to abrogate renal interstitial fibrosis

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Fibrosis is a hallmark of chronic kidney disease, for which there is currently no effective cure. The hormone relaxin is emerging as an effective antifibrotic therapy; however, its mechanism of action is poorly understood. Recent studies have shown that relaxin disrupts the profibrotic actions of transforming growth factor-β1 (TGF-β1) by its cognate receptor, relaxin family peptide receptor 1 (RXFP1), extracellular signal–regulated kinase phosphorylation, and a neuronal nitric oxide synthase–dependent pathway to abrogate Smad2 phosphorylation. Since angiotensin II also inhibits TGF-β1 activity through its AT2 receptor (AT_2R), we investigated the extent to which relaxin interacts with the AT_2R. The effects of the AT_2R antagonist, PD123319, on relaxin activity were examined in primary rat kidney myofibroblasts, and in kidney tissue from relaxin-treated male wild-type and AT_2R-knockout mice subjected to unilateral ureteric obstruction. Relaxin's antifibrotic actions were significantly blocked by PD123319 *in vitro* and *in vivo*, or when relaxin was administered to AT_2R-knockout mice. While heterodimer complexes were formed between RXFP1 and AT_2Rs independent of ligand binding, relaxin did not directly bind to AT_2Rs but signaled through RXFP1-AT_2R heterodimers to induce its antifibrotic actions. These findings highlight a hitherto unrecognized interaction that may be targeted to control fibrosis progression.

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Characterized by an excessive accumulation of the extracellular matrix (ECM), primarily collagen, fibrosis is a universal response to chronic injury and inflammation in the kidney.[1,2] Prolonged exposure to pathological stimuli and/or profibrotic cytokines causes significant disruption to the regulatory processes that control the rate at which the extracellular matrix is synthesized and degraded, where an imbalance between extracellular matrix synthesis and degradation results in excessive collagen deposition at the site of injury.[3] A failure to resolve this process causes significant nephron destruction leading to progressive organ dysfunction and failure, with damage dependent on the extent of fibrogenesis.[1–3] Angiotensin (Ang) II and transforming growth factor-β1 (TGF-β1) are among the most potent cytokines that drive this pathological process.[4,5]

Despite fibrosis being the final common pathway for all forms of renal disease and an inevitable feature of end-stage kidney failure, there are currently no effective treatments to ameliorate the structural and functional changes that it causes. Furthermore, the cellular and molecular events that underlie this process are poorly understood. Thus, the identification of agents that can alter collagen turnover and remodeling to prevent or even reduce the fibrosis that accompanies progressive renal disease is key to both understanding the cellular and molecular pathways involved and to developing novel treatment strategies.

The ovarian and cardiovascular hormone, relaxin, has emerged as a rapid-acting but safe antifibrotic that ameliorates renal fibrosis in several experimental models, regardless of etiology.[6–9] Although clinical trials have recently explored its vasodilatory benefits in acute heart failure,[10] clinical assessment of the antifibrotic potential of relaxin is less well developed. Despite end-stage kidney disease being progressive, it can take a decade to develop, making it particularly difficult to design, fund, and run trials with hard end points. To this end, a thorough understanding of the signal-transduction mechanisms involved in the antifibrotic

actions of relaxin will significantly facilitate the development of novel therapeutic targets for intervention and design of better clinical trials.

Recent studies have demonstrated that human gene-2 (H2) relaxin (the major stored and circulating form of human relaxin) signals through its cognate G-protein-coupled receptor relaxin family peptide receptor 1 (RXFP1) to activate extracellular signal–regulated kinase phosphorylation (pERK)1/2 and a neuronal nitric oxide (NO) synthase (nNOS)-NO-cyclic guanosine monophosphate (cGMP)–dependent pathway in human[11] and rat[12,13] renal myofibroblasts to inhibit TGF-β1 activity, at the level of Smad2 phosphorylation (pSmad2), an intracellular protein that promotes the profibrotic actions of TGF-β1.[11,14] This in turn inhibits TGF-β1-induced myofibroblast differentiation and myofibroblast-derived aberrant matrix/collagen production,[11,12] while allowing for an upregulation of the matrix metalloproteinases (MMP-1/MMP-13, MMP-2, and MMP-9) that are associated with the breakdown of existing collagen.[13] Furthermore, by suppressing the TGF-β1/pSmad2 axis that inhibits iNOS activity in myofibroblasts,[15] H2 relaxin is able to release iNOS, which through higher levels of NO specifically contributes to the MMP-promoting actions of the hormone.[13]

To further understand how H2 relaxin inhibits the profibrotic influence of TGF-β1 in various fibroblast culture models,[11–13,16–19] this study sought to find the points at which H2 relaxin interacts with the well-established Ang II-TGF-β1 system.[20] Ang II is a well-known vasoconstrictor that increases blood pressure, and a potent profibrogenic cytokine.[21] These classical actions of Ang II along with its ability to promote TGF-β1 activity are mediated through the angiotensin type 1 receptor (AT_1R). Conversely, Ang II also negatively regulates TGF-β1 activity and tissue remodeling by acting at the angiotensin type 2 receptor (AT_2R).[22,23] Both AT_1Rs and AT_2Rs are expressed in the kidney. Given our previous findings that H2 relaxin separately inhibits the collagen-stimulatory actions of Ang II or TGF-β1 in other organs,[19] we aimed to examine the interaction between H2 relaxin and the AT_2R to determine how this influences the profibrotic actions of TGF-β1. The experiments were performed in primary renal myofibroblasts *in vitro* and in an experimental model of tubulointerstitial renal fibrosis *in vivo*.

RESULTS

The antifibrotic actions of H2 relaxin are blocked by the AT_2R antagonist PD123319 *in vitro*

Consistent with our previous findings,[12,13] treatment of renal myofibroblasts with recombinant H2 relaxin (100 ng/ml; 16.8 nmol/l) for 72 h promoted ERK1/2 (p42/p44 mitogen-activated protein kinase (MAP kinase)) phosphorylation (pERK1/2), nNOS expression, and nNOS phosphorylation (pnNOS) by 0.8- to 1-fold (Figure 1a) and levels of collagen-degrading MMPs (MMP-9, MMP-2, MMP-13) by 0.85- to 1.3-fold (Figure 1c), while inhibiting TGF-β1 expression, pSmad2, and α-smooth muscle actin (α-SMA) levels (a marker of myofibroblast differentiation) by 0.5-fold (Figure 1b; Supplementary Figure S1 online) (all $P<0.01$ vs. respective values from untreated cells). All these H2 relaxin–induced effects were blocked by the AT_2R antagonist PD123319 (0.1 μmol/l; all $P<0.01$ vs. H2 relaxin treatment), whereas PD123319 (0.1 μmol/l) alone did not affect basal levels of the various parameters measured (Figure 1).

The antifibrotic actions of H2 relaxin are abrogated by the absence of, or blockade of, AT_2R *in vivo*

To substantiate the above findings in isolated cells (Figure 1), *in vivo* studies examined male $AT_2R^{+/+}$ (wild-type) and $AT_2R^{-/y}$ (knockout) mice after unilateral ureteric obstruction (UUO). Their kidneys were assessed at day 2 (when fibrogenesis can be measured) and day 5 after injury (when renal fibrosis is well established). Mice were also pretreated or delayed-treated with H2 relaxin (0.5 mg/kg per day) alone or in combination with PD123319 (3 mg/kg per day). Total kidney collagen concentration (Figure 2a), collagen IV staining (Figure 2b), and collagen I staining (Figure 2c) were all progressively increased in both $AT_2R^{+/+}$ and $AT_2R^{-/y}$ mice after UUO, but were 0.15- to 1.1-fold greater in $AT_2R^{-/y}$ mice compared with UUO-injured $AT_2R^{+/+}$ mice by day 5 after injury (all $P<0.05$ vs. respective measurements from injured $AT_2R^{+/+}$ mice). Immunohistochemically stained sections of $AT_2R^{-/y}$ mice showed an expanded interstitium, with increased deposition of collagen IV (Figure 2b) and collagen I (Figure 2c) by day 5 after injury. Pretreatment and delayed treatment of $AT_2R^{+/+}$ mice with recombinant H2 relaxin significantly reduced both renal collagen concentration (Figure 2a) and collagen IV staining (Figure 2b) by 0.33- to 0.4-fold, and further reduced collagen I staining (Figure 2c) to levels beyond those measured in day 5 (and even day 2) post-UUO animals (all $P<0.05$ vs. respective measurements from day 5 UUO $AT_2R^{+/+}$ mice). These collagen-inhibitory effects of H2 relaxin treatment were completely lost when it was administered to $AT_2R^{-/y}$ mice, or when it was coadministered with PD123319 to $AT_2R^{+/+}$ mice (all $P<0.01$ vs. respective values from H2 relaxin–pretreated and delayed-treated $AT_2R^{+/+}$ mice; Figure 2).

Kidney protein extracts from day 5 UUO controls and mice that were pretreated with H2 relaxin ± PD123319 were further evaluated for additional targets of relaxin activity. Compared with $AT_2R^{+/+}$ mice, the kidneys of day 5 UUO–injured $AT_2R^{-/y}$ mice had 0.5- to 0.6-fold lower pERK1/2, nNOS, pnNOS (Figure 3a), and MMP-13 levels (Figure 3b), but 1- to 1.3-fold increased TGF-β1, pSmad2, and α-SMA (Figure 3b). Pretreatment of $AT_2R^{+/+}$ mice with recombinant H2 relaxin caused a significant elevation in renal pERK and pnNOS (by 1- to 1.7-fold; Figure 3a) and MMP-13 levels (by 1.6-fold; Figure 3b), but reduced TGF-β1 and α-SMA expression, as well as pSmad2 phosphorylation (by 0.5- to 0.6-fold of that in day 5–injured $AT_2R^{+/+}$ mice; Figure 3b; all $P<0.05$ vs. respective values from day 5–injured $AT_2R^{+/+}$ mice). Again, the effects of relaxin were abrogated when it was administered to $AT_2R^{-/y}$ mice or when it was

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