The Antifibrotic Role of Relaxin

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ABSTRACT

Relaxin, a polypeptide hormone of the insulin superfamily, is involved in the promotion of extracellular matrix remodeling. Emerging evidence supports a potential therapeutic role of relaxin in fibrotic diseases including liver. Relaxin has been shown to limit collagen production and promote collagen degradation. It not only prevents fibrogenesis, but also reduces established scarring. Together, these findings suggest that the liver is a target organ of relaxin. Therefore, the purpose of this review is to provide an overview of relaxin, its receptor, and their signaling with a focus on areas of potential translational research on fibrosis with emphasis on liver.

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Introduction

Relaxin was initially found to induce relaxation of pubic ligaments in guinea pigs (Hisaw 1926). In pregnancy it was found to reduce spontaneous uterine contraction by softening of the cervix and elongation of the interpubic ligament and was thought to be a reproductive hormone, being produced in the reproductive tract of many pregnant mammals including the corpus luteum, placenta and uterus. Relaxin was later found to be a promising therapeutic agent to treat scleroderma because application of the hormone increased skin elasticity [1]. Later expression of relaxin and its receptor mRNA demonstrated the intriguing fact that relaxin has a role in numerous physiological processes far beyond reproduction. It promotes tissue remodeling via increased collagen turnover, and shows promise as an anti-fibrotic agent [2-4]. These discoveries of the diverse action of relaxin as well as the presence of relaxin receptors in tissues such as brain, kidney, heart, lung, and liver suggests a broader role of relaxin [5, 6].

The insulin-relaxin family is a group of evolutionary related proteins which possess a variety of hormonal activities. It is postulated that their respective genes had divergent evolution with time. The relaxin-like family of peptides present in humans includes relaxin 1, relaxin 2 (referred as relaxin), relaxin 3, and the insulin like peptides- InsL3, InsL4, InsL5 and InsL6 [1, 7]. These family members are synthesized as prepro-hormones compromised of a B-C-A domain configuration. The C domain peptide is removed to process the pro-hormone to the mature active peptide that consists of two interchain and one intra chain disulphide bonds between highly conserved cysteine residues on the A and B chains. The seven peptides have high structural but low sequence similarity but despite their structural similarity, relaxin and insulin have no common cellular effects (Bennett 2009).

Relaxin family peptide receptors (RXFPs)

Relaxin family peptide receptors were shown to be leucine-rich G-protein-coupled receptors (GPCRs) in 2002 after remaining elusive for many years. The discovery of identifying these cognate receptor was a major breakthrough in relaxin research [8]. The leucine-rich GPCR receptor (LGR) family includes FSH, LH, TSH and relaxin receptors. These receptors were named LGR7 (now relaxin family peptide receptor; RXFP1) [8, 9] and LGR8 (now RXFP2) [10] respectively. These were called LGRs because receptors for relaxin and INSL3 contain leucine rich repeats in the extracellular domain and share 60% homology.

Relaxin, relaxin 3 and InsL3 bind with different affinity to activate leucine rich receptors, RXFP1 and RXFP2.

Relaxin activates both RXFP1 and RXFP2 but InsL3 activates only RXFP3. Relaxin3 activates RXFP1, 3 and 4 but InsL5 activates only RXFP4. Relaxin 3 and InsL5 interact with GPCRs that are unrelated to LGRs. Relaxin 3 and InsL5 activate GPCR135 (now RXFP3) and GPCR142 (now RXFP4) [11, 12]. This review will focus on the recetors and their signaling in context to liver fibrosis.

Distribution and structure of relaxin receptors

In humans, RXFP1 mRNA is expressed in female reproductive organs such as the cervix [13], ovary, uterus, and placenta [8] as suggested by molecular techniques such as northern blotting and RT-PCR. It is expressed in male reproductive organs such as testis and prostate [8] as well as expressed in the nipple and breast of females [13, 14]. RXFP1 mRNA was also identified in adrenal, brain, bone marrow, heart, kidney, liver, lung, muscle, peripheral blood vessels, salivary glands, skin, and thyroid [8]. The RXFP2 mRNA in humans is expressed in brain, bone marrow, kidney, muscle, pituitary, peripheral blood cells, thyroid, testis, and uterus myometrium [8, 15, 16]. In addition to these, rats and mice also express RXFP1 in the oviduct and intestine [17] and express RXFP2 in the ovary and gubernaculum [10, 18].

In normal rat liver, RXFP1 but not RXFP2, is expressed at low levels, but in cirrhotic liver, expression of both receptors increases significantly. Quiescent hepatic stellate cells (HSC) that would normally store lipid droplet but once activated are responsible for liver fibrosis, express low levels of RXFP1 but not RXFP2 in normal state [19] but once activated HSC express both receptors. Thus, with initiation of fibrosis, there is an enhanced expression of both receptors in hepatic stellate cells.

RXFP1 and RXFP2 receptors consists of a relatively large ectodomains that makes up over half of the receptor size and facilitates relaxin binding, this connects with a seven transmembrane spanning region, and an intracellular C-terminal tail making these receptors multidomain protein [20]. Both RXFP1 and RXFP2 contain unique N-terminal low density lipoprotein receptor type A (LDLa) modules. The LDLa module at the N-terminus is connected to ten leucine rich repeats (LRRs) by a short linker. The LRRs are "capped" at each end by cysteine-rich regions that are important to maintain the protein structure of LRRs [21, 22]. The LRRs are connected by another linker to connect to the seven transmembrane helices. The amino acid sequence of the receptors reveals the post translational glycosylation modification to be important for receptor functional maturation and trafficking to cell surface, receptor activation and receptor signaling [18]; these glycosylation occurs at N-terminally and phosphorylation at ten leucine

rich repeats and at several sites in 7 transmembrane and C terminal tail respectively.

Relaxin family peptide receptor signaling cascade

The discovery of relaxin receptors has helped to study its different signaling pathways and its functional role in more depth. The recent discovery of the relaxin cognate receptors, RXFP1 and RXFP2, showed that in spite of relaxin's structural similarity with insulin, it's signaling through RXFPs is unlike insulin signaling through a tyrosine kinase receptor [20]. Relaxin could induce cyclic adenosine monophosphate (cAMP) was demonstrated in the mouse symphysis [23]. Sanborn *et al* further established this relationship between relaxin and increase in cAMP and was later confirmed in rat myometrial cells [24]. More advances in the signaling research lead to a general consent that relaxin binding to RXFP1 and RXFP2 elicits bioactivity by stimulating adenylate cyclase to increase cAMP, this stimulates downstream effector molecules such as PKA [18, 25-27]. In some cell types such as human macrophages (THP-1) and human breast adenocarcinoma cells (MCF-7), recent studies provide evidence that relaxin stimulates a biphasic cAMP response through RXFP1 with an initial phase of cAMP rise by activated by Gαs that lasts 10-15 minutes before inhibition by GαoB [28, 29]. The delayed phase of cAMP increase is through Gαi activation, that releases Gβ subunits, and these subunits then activate phosphatidylinositol 3 kinase (PI3K) to activate and translocate PKCζ to the cell membrane and activate adenylate cyclase for second, delayed round of cAMP production [28-30]. RXFP2 signaling seems not to be biphasic and is contrary to the complex-biphasic signaling of RXFP1. INSL3 or relaxin 2 stimulates RXFP2 to activate Gαs resulting in cAMP accumulation whereas, GαoB and Gβg mediates the inhibition of RXFP2 mediated cAMP accumulation [29].

In addition to the cAMP pathway, there is evidence that relaxin signals through multiple pathways [5, 25]. The diverse signaling of relaxin on different tissues results in various cellular and physiological processes including tissue remodeling, wound healing, cardiac protection, allergic responses and fibrosis [1, 6]. Bani and co-workers showed Relaxin to work through increased nitric oxide generation in guinea pig hearts, human breast cancer cells, mouse uterus and many other organs [31-33]. This nitric oxide production might use either a PI3K activated Akt pathway or IkB inactivation to increase in nitric oxide synthase 2 gene transcription via nuclear factor kB (NFkB) [34]. Relaxin is shown to signal through MAPK in human endometrial stromal cells and epithelial (HeLa cells) [5]. RXFP1 activation of ERK1/2 depends on the cell type. In human uterine cells and THP-1, RXFP1 also initiates

tyrosine kinase activation [35, 36]. In THP1 cells and human pulmonary artery smooth muscle primary culture, ERK1/2 is quickly activated, within 5 minutes of RXFP1 activation [37] whereas in HeLa cells the response is noted after 45 to 90 minutes [38]. Thus, relaxin achieves its pleiotropic effects through activation of multiple pathways in different cell types.

Hepatic Fibrosis

Fibrosis of the liver is excessive accumulation of scar tissue that results from ongoing inflammation and liver cell death that occurs in most types of chronic liver diseases. Nodules, abnormal spherical areas of cells, form as dying liver cells are replaced by regenerating cells. This regeneration of cells causes the liver to become hard [39]. Fibrosis occurs when excessive scar tissue builds up faster than it can be broken down and removed from the liver. Chronic infection with hepatitis, heavy alcohol consumption, toxins, trauma or other factors can all lead to liver fibrosis. As the liver injury progresses, the scarring becomes more extensive, leading to cirrhosis and hence liver failure [39]. Chronic liver disease and cirrhosis was one among the leading causes of mortality in 2010. Mortality due to chronic liver disease and cirrhosis was over 1 % of total in 2010. Later research has also shown that genetic determinants and environmental factors influence the rate of fibrosis progression. Fibrotic scarring changes the normal architecture and function of the liver. Unrestricted scarring progresses to cirrhosis and loss of the hepatic parenchyma due to increases in collagen deposition. This diseased liver includes altered vasculature resulting in portal hypertension, ascites, encephalopathy and finally disruption of metabolic functions of the liver as a whole. In the early stages of liver fibrosis, few people experience symptoms because the liver functions relatively well. Fibrosis is the initial stage of the formation of scar tissue in the liver. An individual may have no symptoms and live a normal, sometimes very active life, for decades, and remain unaware that he or she has liver disease. As scar tissue builds up, due to inflammation and the continuance of liver injury, it connects with existing scar tissue, which can eventually disrupt the metabolic functions of the liver. If the disease progresses, it can lead to cirrhosis, a condition in which the liver is severely scarred, its blood flow is restricted, and its ability to function is impaired. If poked, a healthy liver is very soft. A liver that has developed fibrosis is firmer, and if the condition progresses to cirrhosis, the liver can be almost rock-hard. HSCs are the major contributor to injury-induced collagen production and removal of the causative agent or liver transplantation are currently the only treatments available.

Hepatic Stellate Cells

Hepatic stellate cells, also known as perisinusoidal cells (earlier *lipocytes* or *fat-storing cells*), are pericytes found in the perisinusoidal space of liver [40]. The stellate cell is the major cell type involved in liver fibrosis, which is the formation of scar tissue in response to liver damage. Substantial evidence now exists to recognize HSCs as the main matrix-producing cells in the process of liver fibrosis. Liver injury of any etiology will ultimately lead to activation of HSCs, which undergo transdifferentiation to fibrogenic myofibroblast-like cells. HSCs are major mediators of the fibrotic process in liver during the wound healing process. In normal uninjured liver, HSC are quiescent cells that store vitamin A. As a result of injury, HSCs activate or transdifferentiate to a myofibroblast-like cell that is characterized by having a different phenotype and properties, including loss of normal retinoid-storing capacity, changes in cellular morphology and cytoskeletal organization, enhanced cell migration, adhesion, contractibility and proliferation, expression of α-SMA and acquisition of fibrogenic properties [41-43]. The expression of matrix-degrading enzymes such as matrix metalloproteinases (MMP)-1 and MMP 13 that degrade fibrillar collagen and MMP2 and MMP9 that degrade basement membrane collagen is reduced. Simultaneously, expression of tissue inhibitors of metalloproteinases (TIMPs) is increased [44]. Hence chronic liver injury leads to overall changes in expression of enzymes involved in matrix degradation resulting to a state towards accumulation of collagen and hepatic fibrosis. PPAR agonists and relaxin have been shown to prevent the activation of activated HSC, reversing them back to quiescent stage and thus preventing progressive hepatic fibrosis [9, 45, 46]. Recent studies has also demonstrated that integrin-linked kinase, an intracytoplasmic integrin-associated signaling molecule, has an important role in fibrogenesis. These findings reinforce the hypothesis that activation of HSCs may be triggered not only by circulating and locally released mediators, but also by alterations in the ECM itself, due to direct interaction between these cells and adjacent matrix fiber molecules. On a molecular level, the different morphologic features of fibrosis associated with various etiologies are probably related to the distribution of the primary sites of HSC activation[39]. The stellate cells are also involved in the regulation of portal venous blood flow and have a role in the development of portal hypertension and its complications. There is now strong evidence to support that portal hypertension may be in part caused by modulation of the contractile activity of stellate cells in the perisinusoidal space, which function as liver-specific pericytes and overall have implications in fibrosis [39].

Relaxin and fibrosis

The role of relaxin in fibrosis of the liver has been less studied than that in other organs. The major collagen-producing cell in hepatic fibrosis is the HSCs, a perisinusoidal lipid-storing cell that transdifferentiates into a myofibroblastic cell with liver injury, with increased smooth muscle actin expression, collagen expression and secretion, contractility, and TIMP expression, and decreased MMP expression [47]. Relaxin is responsible for widespread extracellular remodeling of vagina, cervix and in some species, the pubic symphysis during pregnancy [1]. These findings were later confirmed using the relaxin-null mouse model [48]. The relaxin-null mouse developed multiple fibroses with aging, as evidenced by increased accumulation of interstitial collagen. In many cases, this excess collagen accumulation could be reversed by restoring relaxin levels in these animals [49]. Role of relaxin in preventing fibrosis have been examined in many major organs including skin, lung, and heart. Relaxin was reported to decrease the synthesis of TIMP-1 in dermal fibroblasts and enhance the expression of collagenase in culture of dermal fibroblasts [5]. Relaxin was shown to reduce fibrotic lesions and skin thickness, characteristics of scleroderma, by decreasing collagen secretion and increasing collagen degradation [4, 50, 51]. The RXFP1-null and relaxin-null mouse models demonstrate age-associated pulmonary fibrosis [52]. Relaxin treatment of the relaxin null mice that have knocked out relaxin gene and experimental models of lung fibrosis in the mouse model was enough to reverse pulmonary fibrosis [47, 52]. Relaxin inhibits the transition of cardiac fibroblasts to active myofibroblasts, decreases collagen I and III, and increases MMP secretion [53]. Relaxin has also been found to be a potent regulator of collagen in the ECM of heart [53]. Its antifibrotic role have resulted in unprecedented interest in relaxin.

Relaxin has antifibrotic effects in experimental models of renal fibrosis. The relaxin-null mouse is itself a model of progressive renal fibrosis [4]. In hepatic fibrosis, HSCs are the major collagen secreting cells. In activated HSC, relaxin treatment decreased the expression of smooth muscle actin, type I collagen and total collagen and decreased the synthesis of new collagen. Furthermore, relaxin decreased TIMP-1 and TIMP-2 and increased interstitial collagenase levels [46]. The quiescent hepatic cells express RXFP1 at low levels, but not RXFP2. During progression to the activated phenotype, expression of RXFP1 and RXFP2 increases significantly. These changes are also seen in the cirrhotic liver, where expression of the receptors is increased, suggesting the role of these receptors and their ligands during fibrosis [19]. The *in vivo* effect of relaxin treatment on rat CCl_4 model resulted in significant

decrease in liver weight, hepatic hydroxyproline levels and reduction in collagen deposition [54]. Relaxin's effects on fibrotic markers have been found similar to agonists of PPARg. Recently, relaxin have been shown to activate PPARg in HEK293T, THP1 and hepatic stellate cell line (LX2) [9, 26, 27, 55]. The progression of liver fibrosis with increased HSC activation is accompanied with decreased PPAR expression that makes PPAR ligands less useful in advanced liver fibrosis [56]. But in the early stages of HSC activation, combined effect of relaxin and PPARg agonists could be of therapeutic significance in reducing liver fibrosis [9, 26, 27, 55]. More studies are needed to determine if relaxin can improve less severe hepatic fibrosis, or fibrosis induced by less toxic means.

Conclusion

Fibrosis is a leading cause of organ failure and causes loss of organ function when normal tissue is replaced with excess of scarring connective tissue. Several organs are prone to this process regardless of the cause. Relaxin is emerging as an important, naturally occurring inhibitor of collagen turnover in several organs within the body. Relaxin studies have established it as well proven antifibrotic agent that does not appear to influence the extracellular matrix under normal conditions. Relaxin treatment has improved fibrosis in a variety of animal models, suggesting that this may be a valuable area for translational research in fibrosis. Investigating the mechanism of relaxin effects on extracellular matrix and its role in combination with other drugs could advance the field by providing better fibrotic treatments.

Acknowledgements

Competing interests
None declared

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