

Natural agents with antifibrotic properties: The hormone relaxin

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SUMMARY

Relaxin (RLX) is an heterodimeric polypeptidic hormone that belongs to the insulin-like superfamily. Three human genes coding for H1, H2 and H3 relaxin have been identified. In women, the H2 gene is expressed in the corpus luteum, endometrium, placenta, and breast, while H1 mRNA has been found in the placenta only. In men, H1 and H2 expression has been reported in the prostate gland and seminal vesicles. *In vitro* and *in vivo* studies of exogenous RLX administration have shown a substantial reduction in collagen production and tissue metalloproteinase inhibitor-1 and 2 (TIMP-1, TIMP-2) expression by dermal and lung fibroblasts and by hepatic stellate cells. The role of RLX in fibrostenotic Crohn's disease is also under investigation. One of the hypotheses in fibrosis suggests that progression of fibrosis results from increased synthesis of extracellular matrix molecules along with elevated expression of TIMP-1 and 2 which inhibit matrix degradation. Consequently, antifibrotic therapies must target towards either reducing matrix synthesis or/and increasing matrix degradation. These promising data for RLX could be regarded nowadays as a point of interest in trials for every disease that is pathophysiologically linked with fibrotic or fibrostenotic procedures due to abnormal collagen accumulation or collagen degradation.

Key words: relaxin, fibrosis, cirrhosis, liver, hepatitis, collagen

INTRODUCTION

There is a continuously increasing interest in gastroenterology and other medical specialties for the antifibrotic properties of relaxin. The hormone relaxin (RLX) is a disulfidic analogue of insulin, which Hisaw has initially described in 1926¹. The name relaxin is attributed to its reported actions in relaxing and dilating interpubic symphysis and cervical and vaginal tissue just prior to and during parturition². The hormonal activity has been classically detected during pregnancy in plasma and urine extracts³.

Recent *in vitro* and *in vivo* studies have shown that this hormone may have an important role in fibrotic procedures through its intervention in certain regulatory mechanisms of collagen degradation. Moreover, it has been reported that recombinant human RLX administration (exogenous RLX) can improve or even reverse up to a certain level the fibrotic phenomena of several diseases⁴.

Fibrosis is a universal response to chronic injury and inflammation of several organs and manifests itself as an excess accumulation of connective tissue, resulting in an irreversible loss of tissue function when normal tissue is replaced by scar tissue^{5,6}. Fibrosis exists in numerous forms including vascular sclerosis, hepatic cirrhosis, pulmonary fibrosis and renal fibrosis. These forms of deep organ fibrosis are particularly serious because the progressive loss of organ function is a leading cause of mortality estimated to account for 45% of deaths in the United States between 1984 and 1989⁵.

Known for many years as a hormone of human reproduction, the polypeptide RLX has attracted more general attention since the early 1990s, when new studies revealed the remarkable pleiotropy of this substance in rodents^{7,8}. One of the most consistent biological effects of RLX is its ability to stimulate the breakdown of collagen, a major component of all organs within the body. RLX not only

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stimulates collagen remodeling within the birth canal in preparation for parturition, but acts on cells and tissues to inhibit fibrosis, the process of tissue scarring which is primarily the result of excessive collagen deposition.

Thus, the recent promising data for RLX could be regarded nowadays as a point of interest in *in vivo* and *in vitro* research and trials for every disease that is pathophysiologically linked with fibrotic or fibrostenotic procedures due to abnormal collagen accumulation or collagen degradation.

Relaxin production and structure

Relaxin (RLX) is an heterodimeric polypeptidic hormone that belongs to the insulin-like superfamily. This family of proteins includes insulin, RLX, insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) and the relaxin-like factor⁹, that seem to share common evolution origin¹⁰. All members of this family of growth factors appear to be synthesized as a prehormone consisting of a signal peptide and B, C and A domains from the N^o to the C^o terminus respectively^{11,12}. Human RLX is a simple protein of a molecular weight of approximately 6000 daltons¹³. Like insulin, RLX is synthesized as a single chain prohormone that is post-translationally processed by cleavage of a C-peptide region to yield two chains, designated A and B which are connected by inter- and intra-chain disulfide bounds¹⁴. The RLX molecules in different species display significant differences in their nucleic acid and amino acid sequences but always contain a certain amino-acid motif, which has shown to be important for binding to the relaxin receptor¹⁵.

While RLX was one of the first peptides to be discovered, its receptors (LGR7 and LGR8) were only recently discovered^{16,17,18} and found to interact with related G-protein-coupled receptors (GPCRs). The RLX receptors are part of a subgroup (type C) of the family of leucine-rich-repeat-containing GPCRs (LGR). Although LGR7 is more highly responsive to RLX than LGR8, it has been hypothesized that LGR8 acts as a paracrine receptor for locally produced RLX¹⁹.

Humans and higher primates have three RLX genes, designated as H1, H2 and H3 relaxin^{20,26}. In women, the H2 gene is expressed in the corpus luteum, endometrium, placenta, and breast, while H1 mRNA has been found in the placenta only²¹. In men, H1 and H2 expression has been reported in the seminal vesicles and the prostate gland^{22,23}, where it seems that RLX promoters are repressed in contrast with the androgens and the prostatic specific antigen (PSA)²⁴. In human plasma only H2 RLX has been detected²⁵. The H3 RLX gene is predominantly

expressed in the brain where it is thought to act as a neuropeptide²⁶. For many years, RLX had been considered only as a pregnancy hormone but, with improved detection technique, has also been found in nonpregnant women and during the early stages of placentation²⁷. However, the highest levels of RLX are measured in pregnancy and during the second phase of the menstrual cycle²⁸. During menstrual cycle two types of biochemical compounds with hormonal activity are synthesized and secreted by ovarian tissue. One is the polypeptide RLX and the second is a class of compounds with steroidal structure, which includes androgens, estrogens and progestogens. The origin of relaxin in postmenopausal women with atrophic or surgically removed genitals is unknown since the usual sources of RLX, the ovaries and the endometrium are not likely to be the source²⁹.

In animals RLX has also the same important function during parturition. In the pregnant uterus, fetal trophoblast cells have been identified as the source of RLX in the mare, the rabbit and the golden hamster³⁰. In the guinea pig RLX is localized in endometrial glands and in various animal species endometrial granulocytes have also been found to contain RLX³¹. In cats the placenta is the major source of RLX and concentrations of RLX detected in rabbit plasma during the preimplantation period seem to indicate a possible involvement of RLX in implantation in that species. In addition to this, feline RLX is undetectable during estrus cycle and pseudopregnancy and is a valid marker to indicate and monitor placenta sufficiency in feline species³².

Endogenous relaxin physiology and target tissues

The binding of relaxin to specific receptors has been observed in the brain, uterus mammary glands³³ and the heart, which speaks in favor for the multifunctionality of this hormone¹⁹ (**Table I**). In laboratory animals RLX has been shown to cause dilatation of the cervix in mice and rats, a lengthening of the pubic symphysis of mice and guinea pigs and inhibition of uterine contraction in guinea pigs³⁴ and rats³⁵. Additionally, RLX has been shown to have anabolic effects and to induce glycogen accumulation in the myometrium as well as to promote lipid deposition in the adipose tissue³⁵. Furthermore, radioimmunologic (RIA) data confirm that serum level of this hormone in many experimental animals increases significantly within hours of parturition and drops sharply thereafter³⁶.

It has been shown that nerve growth factor (NGF) and RLX have regions considerably different from these of insulin, which may be consistent with the failure of these proteins to exert insulin-like effects³⁷. Moreover, the

Table 1. Target tissues for endogenous relaxin *in vivo*

TARGET TISSUE	EFFECTS
Interpubic ligament	remodeling-lengthening
Vagina and cervix	dilatation and softening
Uterus	inhibition of contraction
Prostate and testicles	development of prostate, sperm maturation and motility
Central nervous system	influence on the bodily fluid balance affecting neurons in the subfornical organ, stimulation of vasopressin and secretion of water drinking
Liver	fibrosis inhibition, glycogenolysis
Heart	vasodilatory, diuretic, chronotropic, inotropic effect
Lung	fibrosis inhibition, reduction of allergic airways inflammation
Kidneys	reduction of glomerular and interstitial fibrosis
Mammary glands and nipples	lactation
Skin	Improvement of extensibility, reduction of dermal scarring
Adipose tissue	Lipid deposition

mRNA for insulin-related receptor is expressed in many tissues including the liver but does not appear to be the receptor of any known members of the insulin family³⁸. Insulin receptors in rat brain cortex are functionally different from other tissues regarding the insulin specificity and no competition was observed with porcine RLX³⁹. Moreover it has been shown that porcine RLX and insulin do not cross react immunologically and do not compete effectively for binding to the same receptors³⁸. It is not known whether hepatocytes express RLX receptors, thus it is not known if this is a result of direct action of the hormone or whether other factors also contribute. The current finding on the RLX-induced glycogen depletion⁴⁰ does not allow defining whether this hormone actually reduces glucose uptake by hepatocytes or rather promotes glycogenolysis. RLX appears to share the ability to maintain adequate levels of glycemia for maternal tissues and metabolic supply to the placenta⁴¹. However, RLX administration did not increase the level of cyclic AMP (cAMP)³⁶ in a non target tissue, other than pubic symphysis, such as liver is. This specific tissue response still remains under investigation.

Reported experiments in nonpregnant animals indicated that RLX influences the cardiovascular system by developing chronotropic and inotropic effects as well as vasodilation and the lowering of blood pressure in normotensive as well as in spontaneous hypertensive rats⁴². The effects were however stronger in comparison to angiotensin II and it has been assumed that this effect is mediated via specific receptors in the heart. RLX interacts also with several neural angiotensinergic pathways in the

central nervous system^{43,44}. Systemic stimuli that may activate central angiotensinergic pathways include plasma hypertonicity and circulating hormones such as angiotensin II and /or RLX.

Little is known about the mechanism(s) by which RLX is degraded. It has been reported that an apparent degradation of rat RLX has been observed in the presence of microsomal membrane fractions of rat uterus⁴⁵. The results of another study⁴⁶ demonstrated that porcine RLX is a viable, although not a preferential substrate for the two out of three of the insulin degrading enzymes; GIT (Glutathione-insulin transhydrogenase) and NTP (NeutraThiol Peptidase). Furthermore, inhibition of insulin degradation by RLX is unlikely to be a significant factor in the availability and kinetics of insulin in tissue.

RLX like insulin is excreted in the urine provided there is normal renal function. An increase in urinary RLX reflected the increase of systemic RLX production after transdermal estradiol treatment, which has been suggested to favor RLX responsiveness in several target organs⁴⁷.

RLX has been reported to be undetectable in the serum of untreated males but extremely sensitive ELISA methods can measure RLX also in non pregnant status (male and female subjects) in serum, plasma, seminal plasma, tissue extract and cell culture⁴⁸.

RLX levels are usually expressed in nanograms per ml (ng/ml). RLX releasing factor⁴⁹ measurement is also achievable with commercial kits but there is not yet adequate information available in order to interpret efficiently the obtained results.

Exogenous relaxin administration *in vitro* and *in vivo*

In vitro studies of exogenous RLX administration have shown a substantial reduction in collagen production and tissue metalloproteinase-1 and 2 (TIMP-1 and TIMP-2) expression by dermal and lung fibroblasts⁵⁰ (Figure 1). Many experimental studies have also shown the RLX effect on structural remodeling of the liver⁵¹, cervix and interpubic ligament in preparation for parturition¹⁹.

Exposure of activated rat hepatic stellate cells (HSC) to RLX (100 ng/ml) resulted in a concentration dependent (dose-dependent) decrease in both collagen synthesis and deposition⁵². In detail, a 40% decrease in collagen synthesis was noticed in a 24-hour period of observation. These data demonstrate that RLX modulates effective collagen deposition by HSC, at least in part, due to changes in the pattern of matrix degradation. In this study the authors have proposed that RLX regulates collagen deposition at the translational or post-translational level in the hepatic cells.

The effect of RLX in HSC was also studied in an *in vivo* rat model of liver fibrosis (using CCL4)⁵³. In this model subcutaneous infusion of recombinant RLX in much more excessive levels than in normal pregnant women resulted in a significant reduction of the effective deposition of interstitial collagen. Treatment with RLX led to a decrease in liver size associated with a significant decrease in total liver hydroxyproline content. This antifibrotic effect of RLX was comparable with that seen in the rodent model of pulmonary fibrosis but less dramatic than that observed with the rodent dermal fibrosis. However, this study did not clearly show the exact mechanism of how RLX acts with an antifibrotic way in the rat liver. Reduction of collagen synthesis resulted in

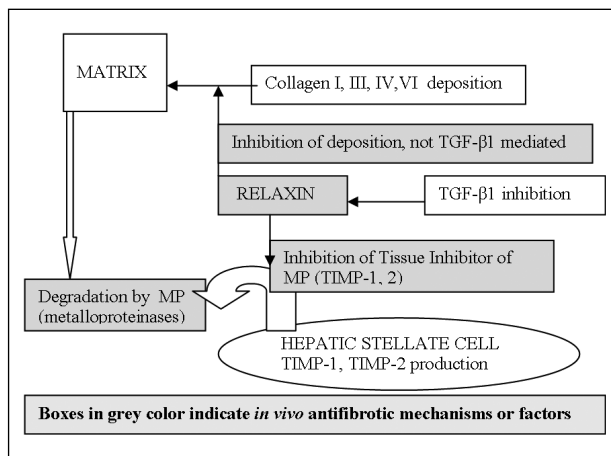


Figure 1. The probable antifibrotic role of relaxin in the liver

a modest, although consistent, decrease in TIMP-mRNA levels. On the other hand, secreted substances from HSC such as collagenase, MMP-13 and gelatinase A and B, although enhanced, were not RLX mediated in the same experimental study.

In addition, in other studies RLX treatment of rats has been reported to cause acute changes in hepatic microcirculation and to induce morphological changes in sinusoidal myofibroblastic cells⁵⁴, dilation of the sinusoids, decreasing also the expression of tissue inhibitors of metalloproteinases (TIMPs) and increasing the expression of the rodent interstitial collagenase MMP-13⁵⁵. RLX had also protective effects against ischemia and reperfusion damage in perfused rat liver⁵⁶, while promoting vasodilation, wound healing and angiogenesis⁵⁷. Finally, RLX treatment of experimentally induced hepatic fibrosis resulted in decreased liver collagen, suggesting that RLX treatment may benefit hepatic fibrosis *in vivo*⁵⁸.

From the body of data obtained by investigating the effects of exogenous RLX in rodents, we may predict that this peptide chiefly exerts compensatory effects in the course of congestive heart failure⁵⁹, vasodilation, diuresis caused by attenuation of the renal vascular response to angiotensin-II^{60,61}, stimulation of atrial natriuretic peptide^{61,62}, collagen matrix degradation, prevention of coronary thrombotic events by upregulating tissue plasminogen activator, modulation of angiotensin-II effects and suppression of the endothelin-1 system⁶². Intravenous or intracerebroventricular (ICV) RLX administration in rats resulted in increased circulating levels of vasopressin and significant increase in water intake probably as a result of central nervous system angiotensin receptor modulation^{63,64}. Administration of RLX in rats causes widespread glycogen depletion as well as increase in the organelles involved in the uptake of blood-borne substances and their metabolism such as smooth endoplasmic reticulum, endosomes and lysosomes⁴⁰.

Recombinant human RLX reduced collagen accumulation in a rodent model of dermal fibrosis⁶. RLX was also associated with a significant reduction in a bleomycin induced murine model of lung fibrosis⁵⁰. In a rodent model of renal fibrosis the anti-fibrotic role of exogenous RLX administration has also been reported⁶⁵⁻⁶⁶. RLX administration was also suggested for the treatment of systemic sclerosis⁶⁷⁻⁶⁸. In addition, in a recent study conducted by Samuel *et al*⁶⁹ it was demonstrated that mouse RLX can effectively inhibit collagen deposition and accumulation over long-term treatment periods (>4 months) in several organs including the lung, kidney, testis and skin.

The observation that pregnancy decreases the incidence of relapses and the development of fibrostenotic

lesions in women with Crohn's disease could be of great importance. RLX in multiparous women with Crohn's disease increases the laxity of fibrous tissue and probably enhances remodeling of fibrostenotic bowel lesions^{70,71}. Glycoprotein YKL-40, which causes fibrosis in rheumatoid arthritis and cirrhosis, is speculated to be lower in multiparous women than in nonpregnant women due to the fetal lymphocytes that secrete a protein that is a potential immune modulator⁷¹⁻⁷³.

Future perspectives of exogenous relaxin use in gastroenterology

Liver fibrosis is characterized by increased hepatic deposition of extracellular matrix (ECM) proteins, fibril-forming collagens (types I, III and IV), some non-fibril forming collagens (types IV and VI), a number of glycoproteins (cellular fibronectin, laminin, SPARC, osteonectin, tenascin and von Willenbrand factor), proteoglycans and glycosaminoglycans (perlecan, decorin, aggrecan, lumican and fibromodulin)⁷⁴⁻⁷⁶. The hepatic stellate cell (HSC) is the main cell involved in hepatic fibrogenesis. After liver injury or inflammation, HSC become activated, proliferated and undergo transformation to a myofibroblast-like phenotype. Activated HSC expresses mostly type I and III collagen, matrix degrading metalloproteinases, the TGF- β 1, tissue metalloproteinase 1 and 2 inhibitors (TIMPs)^{77,78} and their downstream mediators including connective tissue growth factor (CTGF), platelet derived growth factor (PDGF) and endothelin-1 (ET-1). Furthermore, an imbalance between collagen degrading enzymes, the matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), can also lead to excessive collagen deposition and result in liver fibrosis (**Table 2**). The irregular deposition of matrix material leads to the disruption of normal liver architecture (cirrhosis) and eventually to organ dysfunction.

RLX was shown to act on HSC in a two-pronged manner, first by reducing markers of HSC activation (reduced production of collagen and smooth muscle actin by HSC) and second by promoting fibrillar matrix degradation by elevating collagenase activity⁵⁶. The result is a shift in the HSC from a phenotype that contributes to fibrosis to one that promotes recovery. Furthermore, in the recovery stage of experimental hepatic fibrosis a large induction of MMP-13 expression was detected, suggesting that MMP-13 is an integral part of the antifibrotic process⁷⁹. RLX was found to increase the expression and secretion of MMP-13⁵⁶.

In recent studies conducted by Bennett *et al*^{80,81} the expression of LGR7 and LGR8 in activated HSC and in the cirrhotic rat liver increased markedly, whereas in the nor-

Table 2. Proposed antifibrotic mechanisms of exogenous relaxin administration.

Proposed antifibrotic mechanisms during exogenous RLX administration (rat model)

1. Down regulation of collagen synthesis and α -smooth muscle actin (SMA)
2. Down regulation of TIMP-1,2 synthesis by hepatic stellate cells (HSC)
3. Down regulation of the TGF- β 1, AngII, PDGF, ET-1, CTGF
4. Reduction of TIMP-1 mRNA expression
5. Enhancement of matrix degradation by metalloproteinases
6. Increase of collagenase and proteoglycanase production and effects

mal rat liver neither receptor was detectable. Therefore, the authors suggest that agents stimulating both receptors may be therapeutically useful to limit the activation of HSC in liver injury and to modify fibrosis.

In addition, antifibrotic therapies of benign and also probably malignant diseases such as scirrhus carcinoma of the stomach^{82,83} must target towards either reducing matrix synthesis or/and increasing matrix degradation. Translating experimental findings to humans will however be essential in determining the clinical significance of relaxin. Thus, further clinical trials aiming to validate its significance in human pathology are therefore mandatory.

In conclusion, RLX is emerging as a potent antifibrotic agent with rapid occurring efficacy and excellent safety through its ability to regulate the ECM and in particular collagen turnover at multiple levels. It not only prevents fibrogenesis but also reduces established scarring (fibrosis) which is a leading cause of organ failure and affects several tissues regardless of etiology. Especially the role of RLX in the treatment of liver fibrosis disease seems promising.

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