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Relaxin and the extracellular matrix: molecular mechanisms of action and implications for cardiovascular disease

Jonathan T. McGuane and Laura J. Parry

Myocardial fibrosis is a common endpoint in a variety of cardiac pathologies. It results from excessive accumulation of collagen and other materials that together comprise the extracellular matrix (ECM). In the past decade, the peptide hormone relaxin has emerged as an important regulator of the ECM within several organs, including the heart, and has been suggested as a novel therapeutic agent for the treatment of fibrotic disorders. This review summarises research on the anti-fibrotic actions of relaxin, outlines the potential mechanisms by which relaxin regulates the ECM in cardiovascular tissues and examines the implications of this research for the management of heart disease. Some of the contradictions in the literature are also addressed in order to clarify the role of relaxin as an anti-fibrotic factor in vivo.

Myocardial fibrosis is a serious complicating factor in many diseases of the cardiovascular system and effective treatments against the adverse accumulation of fibrillar collagen in the heart are readily sought. The peptide hormone relaxin has emerged as a potential therapeutic in the treatment of ventricular fibrosis. Significant advances in relaxin research include the identification of several receptors for relaxin and related peptides, and the development of relaxin-deficient mice. This review summarises the composition and regulation of the extracellular matrix (ECM) in the heart, and discusses possible mechanisms involved in the development of myocardial fibrosis. In addition, the evidence for relaxin as a cardiac hormone and a key mediator of the ECM is outlined. Recent controversies concerning the ability of relaxin to 'break down'

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collagen fibres are also discussed, as are a variety of cardiovascular diseases that could be targeted for relaxin treatment.

ECM

The ECM is the complex network of macromolecules that provides the architectural framework for all tissues and organs of the body, including the cardiovascular system. It functions to absorb mechanical stress and provide tensile strength to tissues, but can also influence cellular development, migration and proliferation (Ref. 1). In the heart, the ECM allows transduction of mechanical force and maintains capillary patency and cellular alignment during ventricular contraction (Ref. 2). The opposing processes of degradation and synthesis dictate the composition of the cardiac ECM, although the overall content remains relatively homeostatic under normal physiological conditions. However, synthesis and turnover of ECM macromolecules can be altered rapidly in response to physiological or pathological stimuli.

Constituents of the ECM

The ECM consists of a variety of structurally and functionally divergent molecules including glycosaminoglycans, fibronectin, fibrillin, elastin and collagen (of which there are several isoforms). These are differentially expressed depending on tissue type and physiological state. Undoubtedly, the most important ECM component is collagen, which in mammals accounts for ~25% of the total protein mass, making it the most abundant protein in the body (Ref. 3). Fibrillar collagen molecules are synthesised as pro-α chains, which wind around each other to form a triple-stranded helix. After secretion into the extracellular space, these helices self-assemble into fibrils of 10–300 nm in diameter, which then aggregate into fibres ranging 0.5–3 μm in diameter. The development of covalent crosslinks strengthens the macromolecular structure of fibrillar collagen and partly characterises it as 'mature' (Ref. 3). Type I and III collagens are the main subtypes in the heart, comprising approximately 85% and 11% of total cardiac collagen, respectively (Ref. 4). Fibres containing type I collagen are usually thick (average diameter 75 nm) and have high tensile strength, whereas type III collagen tends to be associated with thinner fibres (average diameter 45 nm) (Ref. 4). Myocardial function, and thus cardiac performance, is influenced not only by the amount of collagen but also by the proportions of collagen types represented and the extent of collagen crosslinking.

ECM synthesis

It is generally accepted that the cell types primarily responsible for de novo synthesis of the ECM are fibroblasts and/or myofibroblasts (Ref. 5), the latter being smooth muscle-like cells thought to derive from pre-existing fibroblasts (Fig. 1) (Ref. 6). Several factors have been implicated in the induction of this differentiation, including mechanical tension/force (Ref. 7) and 'pro-fibrotic' cytokines such as transforming growth factor β1 (TGF-β1) (Ref. 8) and interleukin 4 (IL-4) (Ref. 9). There is also evidence that the progression of fibroblast differentiation in vivo occurs in discrete steps, which can be defined by the sequential expression of myofibroblastic markers (Refs 5, 6, 10, 11). It is not known if these intermediate steps are obligatory or identical for fibroblast differentiation in all tissues. Interestingly, it has been suggested that myofibroblasts themselves might represent an intermediate phenotype in a differentiation spectrum from fibroblasts to smooth muscle cells (Ref. 7), of which there may also be 'quiescent' and 'ECM-synthesising' phenotypes (Ref. 12). Regardless, several characteristic features that distinguish myofibroblasts from fibroblasts are acquired during differentiation: the actin cytoskeleton is reorganised and enhanced, and α-smooth muscle actin-containing 'stress fibres' become prominent. This modified cytoskeletal network, in association with membrane proteins (e.g. integrins, vinculin and paxillin) and extracellular fibronectin fibrils, forms a transmembrane apparatus that intimately connects the cytosolic and interstitial spaces. These connections confer enhanced intracellular contractility upon the myofibroblast, and the ability to induce tension in the surrounding ECM (Ref. 7). Although the heightened capacity of myofibroblasts for ECM production is probably the most important feature with respect to fibrosis, this phenotypic shift of the mesenchymal cell population might also contribute directly to altered mechanical functionality of contractile tissues such as the heart.

ECM degradation

It is beyond the scope of this review to detail the post-translational regulation of the ECM,

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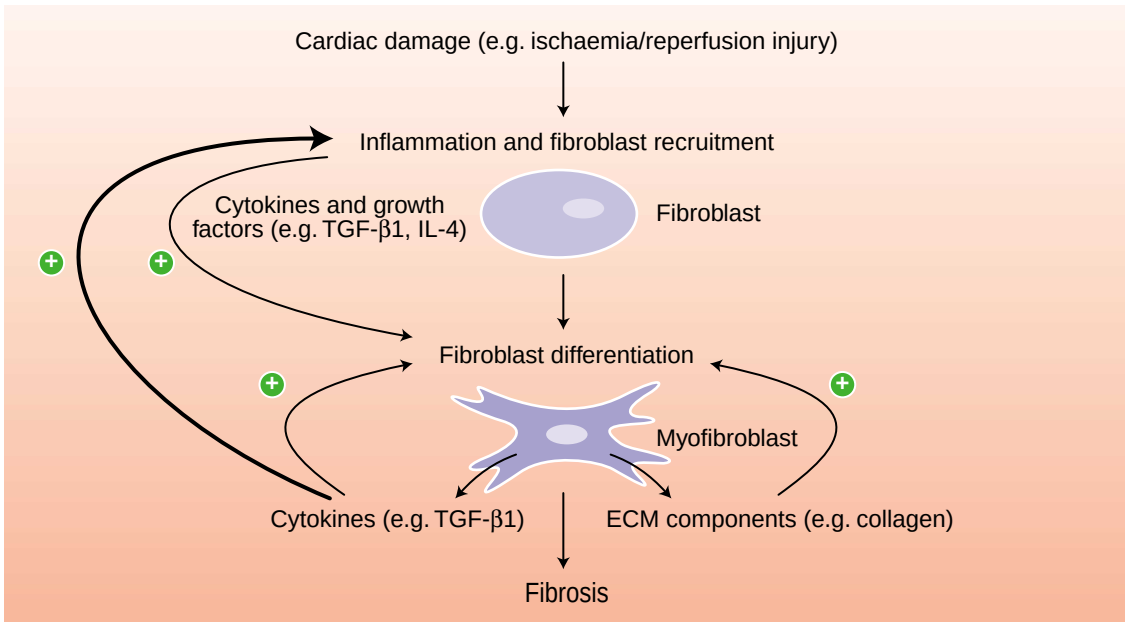

A generalised scheme of cardiac fibrosis

Figure 1. A generalised scheme of cardiac fibrosis. An inflammation phase immediately follows injury and precedes active fibrosis. Cytokines and growth factors [e.g. transforming growth factor β1 (TGF-β1) and interleukin 4 (IL-4)] stimulate differentiation of fibroblasts to a myofibroblast phenotype during the reparative phase. Further release of cytokines and growth factors from the myofibroblast enhances the inflammatory response and maintains the myofibroblast phenotype. Similarly, the mechanical influence of enhanced extracellular matrix (ECM) deposition and organisation on fibroblasts promotes fibroblast differentiation (Ref. 7). These positive-feedback loops lead to increased production of ECM components, including collagen.

but degradation is one essential aspect. ECM degradation occurs in vivo through the actions of many proteolytic enzymes, whose expression and activity vary considerably in different tissues and organs of the body. The most important group of enzymes with this function is the matrixin or matrix metalloproteinase (MMP) family, of which there are at least 24 members currently recognised in humans (Ref. 13). Several of these enzymes have been designated as 'collagenases' (MMP1, MMP8 and MMP13 in humans; and MMP8 and MMP13 in rodents). Whereas MMP13 is the major collagenase in mice and rats, MMP1 appears to have acquired this function in humans. Collagenases cleave fibrillar collagen at a specific locus, yielding two fragments approximately three-quarters and one-quarter the length of the original collagen chains (Ref. 14), and these fragments may be further catabolised by gelatinases (MMP2 and MMP9). MMP activity is tightly controlled at multiple levels. In addition to regulation of transcriptional status and zymogen activation (most MMPs are secreted as zymogens, or inactive precursors), MMP activity is further controlled by specific endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (Ref. 15). Thus, the overall composition of the ECM is the product of interactions between proteases (MMPs), activators of these proteases, inhibitors (TIMPs) and ECM components themselves, all of which are profoundly influenced by mechanical and hormonal (both systemic and local) cues.

Cardiac fibrosis

Fibrosis is the abnormal deposition of ECM, which causes disruption of normal tissue architecture and may lead to organ dysfunction (Fig. 1). Acute fibrosis occurs after inflammation following tissue

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damage or injury (Refs 16, 17), particularly in conjunction with the post-myocardial infarct (MI) elaboration of inflammatory cytokines (Ref. 18). These include tumour necrosis factor α (TNF-α), IL-1 and IL-6 (Ref. 18), which originate from neutrophils, macrophages and lymphocytes at the site of injury (Ref. 19). Fibroblasts are recruited from the wound margins and are stimulated to differentiate and synthesise ECM (Ref. 20). As the tissue healing process nears completion, ECM deposition decreases and myofibroblasts are removed from the injury site by apoptosis (Refs 6, 21), leaving minimal fibrotic scarring. The precise nature of the stimulus for the induction of myofibroblast apoptosis in vivo is unknown, although loss of TGF-β1-activated intracellular signalling has been implicated (Ref. 11). Interestingly, TGF-β1 prevents nitric oxide (NO)-mediated myofibroblast apoptosis by suppressing inducible nitric oxide synthase (iNOS) induction and maintaining expression of the anti-apoptosis protein Bcl-2 (Ref. 22). In pathological states leading to fibrosis, myofibroblasts persist and ECM deposition continues unchecked (Refs 7, 23). In addition, cytokines produced by myofibroblasts may intensify inflammation by contributing to the recruitment of 'inflammatory' cells, or promote additional fibroblast differentiation and ECM production (Ref. 11). Thus, a positive-feedback loop is created that prolongs the period of active ECM deposition, resulting in fibrosis (Fig. 1). Fibrosis of the ventricular myocardium results in increased tissue stiffness, which can have serious consequences on contractile function.

As well as being associated with inflammation, cardiac fibrosis can also result from other situations. For instance, myocardial fibrosis occurs with aging, through increases in the thickness, content and intermolecular crosslinking of collagen (Ref. 4). Hypertrophic states that develop in response to increased cardiac load or hypertension are often accompanied by ventricular collagen accumulation. Cardiac hypertrophy is stimulated by neurohumoral factors such as norepinephrine, which synergises with TGF-β1 to enhance cardiac fibroblast proliferation and type I collagen protein production in vitro (Ref. 24). Similarly, phenylephrine induces expression of connective tissue growth factor [a pro-fibrotic cytokine that is thought partly to mediate the response of fibroblasts to TGF-β1 (Ref. 23)] in neonatal rat cardiomyocytes (Ref. 25). The phenomenon of cardiac fibrosis is well documented in the spontaneously hypertensive rat model (Refs 26, 27, 28). Finally, fibrosis might also be a key component of diabetic cardiomyopathies. A recent in vitro study showed that high glucose conditions stimulate collagen synthesis in cardiac fibroblasts, an effect that appears to depend on angiotensin II (AII) (Ref. 29). AII itself is a well-known fibrogenic mediator in the myocardium (Ref. 30), and many of the drugs currently used to treat myocardial fibrosis are directed against components of the angiotensin–aldosterone hormone pathway (Ref. 31).

ECM remodelling in the acute phase after MI has become a particularly important target for therapeutic intervention (Ref. 32), as extensive structural modifications of the ventricle are initiated by the injured heart after ischaemia/reperfusion injury (Ref. 33). The intent of this remodelling is to preserve cardiac integrity and function, and ECM deposition in the infarct zone of the ventricle post-MI is essential to prevent dilatation (Ref. 34). Transgenic mice with cardiac-specific overexpression of β_2-adrenergic receptor (β_2AR) show a reduction in the incidence of cardiac rupture following MI, which has been attributed to increased total collagen content of the ventricle (Ref. 35). This is despite an increased heart rate and contractility in β_2AR-overexpressing mice post-MI compared with wild-type mice, emphasising the importance of ventricular collagen in preventing rupture (Ref. 35). Other studies have shown that MMP deletion or inhibition post-MI preserves cardiac contractility and function (Refs 36, 37, 38), suggesting an important role for MMPs in post-MI remodelling. Moreover, TIMP1-deficient mice have increased left-ventricular end diastolic pressure (LVEDP) two weeks post-MI in association with ventricular hypertrophy and loss of fibrillar collagen (Ref. 39). However, termination of ECM deposition is crucial to prevent progression into pathological fibrosis. ECM accumulation (particularly in the non-infarcted myocardium) ultimately results in increased tissue stiffness and might worsen the ventricular dysfunction (Ref. 40). This reactive fibrosis is distinct from the essential reparative fibrosis occurring in the infarct area. Treatment with MMPs might help to prevent or ameliorate this deposition of ECM. However, MMP activity is associated with an increased risk of cardiac

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rupture (Ref. 41), therefore the timing of such intervention is likely to be a critical issue.

In summary, evidence suggests that the benefits of acute post-MI MMP inhibition outweigh the potential deleterious effects of ECM accumulation, although the effects of long-term MMP inhibition are relatively unknown. In the long-term, preventing collagen deposition by upregulating MMPs might in fact be a better strategy, at least after the acute phase. Regardless, it is clear that the balance of ECM synthesis and degradation in cardiac pathologies must be tightly controlled for an optimal outcome.

Relaxin and the relaxin receptor

Relaxin has emerged as a novel regulator of the ECM within several organs and more recently in the cardiovascular system (Refs 42, 43). The hormone is a member of the insulin and insulin-like family of peptide hormones and growth factors (Ref. 44). It was discovered in 1926 after Hisaw observed a relaxation of the pubic ligament in virgin guinea pigs after post-estrus injection of serum from pregnant guinea pigs or rabbits (Ref. 45).

In humans, there are three relaxin genes. The genes for human 1 (H1) and 2 (H2) relaxins are expressed mainly in reproductive tissues, including the ovary, placenta, decidua and prostate gland (Ref. 46). H2 relaxin is thought to be the predominant circulating form in humans (Ref. 44), whereas transcripts of the gene for H3 relaxin have only been detected by RT-PCR in the brain and testis (Ref. 47). The rat and mouse genomes encode two of the three known relaxin genes, homologous to H2 and H3 relaxins (Refs 48, 49). In this review, H2 relaxin and the equivalent rodent peptides are referred to as relaxin, and H3 relaxin and its homologues are referred to as relaxin-3. Other members of the insulin and insulin-like family of peptides, such as Leydig-cell insulin-like factor (INSL3), are not widely expressed in non-reproductive tissues and will not be discussed further here.

Relaxin is synthesised as a prohormone, consisting of a B–C–A peptide configuration as shown in Fig. 2. The connecting C peptide is cleaved during processing of the propeptide by prohormone convertases (Ref. 50). The A and B peptides contain highly conserved cysteine residues that form inter- and intra-domain disulphide bonds to link the two peptide chains. To date, the prorelaxin-3 molecule has not been isolated from any tissue. However, based on the

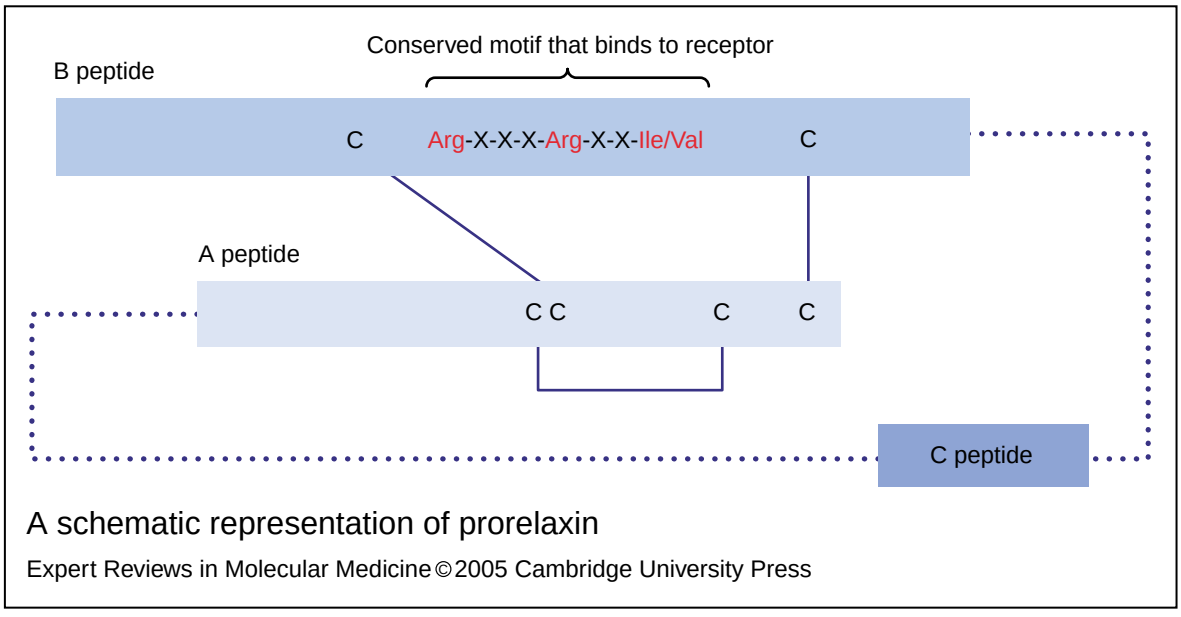

Figure 2. A schematic representation of prorelaxin. Relaxin is synthesised as a prohormone, consisting of a B–C–A peptide configuration The connecting C peptide is cleaved by prohormone convertases, and the B peptide and A peptide are linked by inter- and intra-domain disulphide bonds at the highly conserved cysteine (C) residues to form mature relaxin. Relaxin hormones contain an amino acid motif Arg-X-X-X-Arg-X-X-Ile/Val located in the B peptide, which is the region of the ligand that binds to the receptor and is essential for bioactivity (Ref. 51).

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putative amino acid sequence obtained from the coding region of the gene, the prorelaxin-3 C peptide is much shorter than prorelaxin (Table 1) (Refs 48, 50), the significance of which is unclear. Relaxin hormones contain the amino acid motif Arg-X-X-X-Arg-X-X-Ile/Val located in the B peptide, which is the region of the ligand that binds to the receptor and is essential for bioactivity (Ref. 51). In general, the amino acid sequence homology between relaxin and relaxin-3 is low (~40%). As in humans, rodent relaxin is mainly secreted by the corpus luteum in pregnant animals (Ref. 44), but is also expressed in several peripheral tissues (Ref. 48). Rodent relaxin-3 expression has been localised to a specific region of the brain pons known as the nucleus incertus (Ref. 52). Its selective and high expression in this brain region suggests that relaxin-3 acts as a neuropeptide and does not have a functional role in systemic organ physiology. However, relaxin-3 transcripts have been demonstrated by RT-PCR in a broad range of mouse tissues (Ref. 48), although other studies have not been able to confirm this finding (Refs 47, 53).

The receptors for relaxin proteins are LGR7 and LGR8. These are unique members of the G-protein-coupled receptor (GPCR) superfamily, characterised by a large ectodomain containing an N-terminal low-density lipoprotein (LDL) receptor cysteine-rich motif and ten leucine-rich repeats (Ref. 54). Activation of relaxin receptors by their ligands stimulates a G_s–cAMP–protein kinase A (PKA)-dependent signalling pathway (Refs 54, 55). Pharmacological studies using human 293T cells have demonstrated that relaxin binds to both LGR7 and LGR8 and stimulates cAMP, whereas INSL3 only activates LGR8 (Ref. 56). Both relaxin and INSL3 stimulate an increase in cell proliferation in vitro in the rat gubernaculum (tissue that connects the fetal testis to the scrotum and plays a role in testicular descent) (Ref. 57), implying that relaxin can produce an equivalent biological response to INSL3 by activating LGR8, the only relaxin receptor expressed in the gubernaculum. However, endogenous relaxin does not compensate for the loss of INSL3 in the INSL3-knockout mouse, as shown by the testes not descending (Ref. 58). Thus, although there is some overlap in the specificity of relaxin ligands for the two receptors in vitro, this does not seem to be the case in vivo.

The cardiovascular relaxin system

A central controversy in this field of research is the source of relaxin within the heart. Evidence for circulating relaxin has not been demonstrated convincingly in normal healthy males (with the exception of the boar) or in non-pregnant females other than humans (Ref. 50). It is therefore assumed that a paracrine relaxin system is present within the mammalian heart and vascular tissues (Ref. 42). Relaxin gene transcripts have been identified by RT-PCR in the atria and ventricles of aged male mice (Ref. 59) and in human atrial and ventricular tissues (Ref. 60). Confirmation of relaxin expression in the human heart was provided by western blot analysis, but only prorelaxin peptide was detected (Ref. 60). Plasma relaxin concentrations also increase 4–16-fold in patients with congestive heart failure (CHF) in accordance with the severity of disease (Ref. 60). These data suggested the potential for using relaxin as a marker for monitoring the progression

Table 1. A comparison of the human relaxins

	Number of amino acids			Chromosome location	Molecular weight (Da)
	B peptide	A peptide	C peptide		
H1	28	24	102	9p24.1	5968.2[a]
H2	29	24	102	9p24.1	5962.2
H3	27	24	66	19p13.2	5498.4[b]

[a] Note that the mature form of H1 relaxin is unknown, so the expected molecular weight of the predicted peptide is given.
[b] The H3 relaxin data are based on solid phase chemical synthesis and matrix-assisted laser desorption/ionization (MALDI) time-of-flight (MALDI-TOF) mass spectrometry (Ref. 48).

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of CHF. However, other studies examined circulating relaxin at rest and after physical exercise in CHF patients, and found that serum relaxin concentrations did not differ from control patients (Ref. 61). There was also no difference in systemic relaxin concentrations between control and heart failure patients with or without aortic valve stenosis (Ref. 62), although this study did support the suggestion of local release of relaxin into the circulation in heart failure patients. There was also no correlation between plasma relaxin and any index of cardiac function (Ref. 62).

Recent reports suggest that the rat is an anomaly because it does not appear to express an H2 relaxin homologue in the heart (Refs 63, 64). However, a low level of relaxin-3 expression was reported in adult rat atrium and ventricle (Ref. 63), as well as in atrial myocytes and fibroblasts, ventricular myocytes and fibroblasts, and vascular smooth muscle cells (Ref. 64). Furthermore, in post-MI rats, relaxin-3 expression is upregulated in the atria but not in the left ventricle (Ref. 63). This suggests that relaxin-3 is the predominant relaxin isoform in the rat heart. However, earlier studies did report the presence of relaxin gene transcripts in the rat heart by RT-PCR (Ref. 65) and immunoreactive relaxin secretion by rat atrial myocytes (Ref. 66), whereas other workers have been unable to demonstrate relaxin-3 gene expression in the hearts of either rats or mice (Ref. 53). Thus, the source and identity of functional relaxin peptides in the heart remains unclear, particularly in the rat.

The location of relaxin receptors in the heart is relatively well established. Early work described relaxin-binding sites in male and female rat atria as early as 1 day postpartum, but binding sites were absent from the ventricles (Refs 67, 68). Functional and autoradiographical studies also clearly identified the atria as a target for relaxin in the adult rat heart (Refs 69, 70). More recently, LGR7 gene transcripts were reported in the human and rat heart (Refs 54, 55), and in atrial and ventricular cells isolated from neonatal rats (Ref. 64). Recent studies also localised LGR7-specific β-galactosidase activity in atrial myocytes of 12-week-old heterozygous *LGR7*$^{+/-}$ mice, confirming the predominance of relaxin receptors in the atria (Ref. 71). LGR7 expression was reported to decrease in both the atria and left ventricle after MI in rats, although this assessment was not quantitative (Ref. 63). The current pharmacological data on rat LGR7 provide some interesting perspectives on relaxin function in the rat heart. If relaxin-3 is the 'cardiac relaxin' in rats, it may not act through LGR7. Relaxin-3 binds with relatively low affinity (IC_{50} 32.9 + 9.1 nM) to LGR7 expressed in human 293T cells compared with relaxin (IC_{50} 0.09 + 0.12 nM) and does not stimulate cAMP with the same potency (Ref. 72). Two specific receptors for relaxin-3 have recently been identified as GPCR135 and GPCR142 (Refs 47, 73). The first reports on the expression of these receptors in humans or rodents have indicated (by RT-PCR) that GPCR135 is predominantly expressed in the brain and testis, but not in the heart (Ref. 47). Furthermore, GPCR142, which is a pseudogene in rats, is not expressed in the mouse heart (Ref. 73), and might in fact be the endogenous receptor for insulin-like peptide 5, another member of the insulin and insulin-like family of proteins (Ref. 74). Thus, it is unclear if relaxin-3 in the rat heart interacts with LGR7 or with an alternative receptor.

In summary, it seems likely that one or more relaxin peptides are expressed in the mammalian heart, in addition to the LGR7 receptor. The effect of cardiovascular disease on the secretion of relaxin peptides from the heart or on relaxin receptor expression requires clarification. Furthermore, it is not clear whether or not relaxin-3 binds to and activates atrial LGR7 receptors or another member of the relaxin receptor family elsewhere in the heart.

Relaxin-deficient mice

The generation of a relaxin-knockout (*Rlx*$^{-/-}$) mouse by targeted gene deletion (Ref. 75) has provided insights into the effects of relaxin deficiency within the cardiovascular system. In such mice, blood pressure and contractile function of the heart remained unchanged, but atrial hypertrophy and increased LVEDP were observed in aged *Rlx*$^{-/-}$ males. Diastolic function of the left ventricle was also altered in males aged between 8 and 9 months, with a significantly higher late-filling flow velocity (A-wave) in *Rlx*$^{-/-}$ mice (Ref. 59). These data were largely explained by an accumulation of collagen in the left ventricle of *Rlx*$^{-/-}$ mice compared with wild-type (*Rlx*$^{+/+}$) mice of similar age, which presumably leads to increased ventricular chamber stiffness. It would appear that increased expression of collagen in the myocardium with age is exacerbated by the absence of relaxin, resulting in higher cardiac collagen content and mild dysfunction (Ref. 59).

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These studies also showed that the differences in atrial weight and LVEDP are restricted to male mice, implying the influence of sex-specific factors in relaxin cardiac physiology. This pattern of sexual dimorphism is a recurring theme in genetically modified mouse models of cardiac dysfunction (Ref. 76).

Molecular mechanisms of relaxin action in the heart

ECM production

The principle mechanisms of relaxin action within the cardiac ECM are likely to involve either the direct interaction with fibroblasts to reduce de novo collagen synthesis or the activation of factors that degrade fibrillar collagen. This is based partly on studies using human dermal and lung fibroblasts, both of which respond to treatment with recombinant human relaxin (rH2) by decreasing TGF-β1-stimulated synthesis of collagen (Ref. 77), fibrillin 2 (Ref. 78) and fibronectin (Ref. 79). In the case of fibronectin, relaxin attenuates TGF-β1-stimulated expression of this ECM protein through stimulation of the ubiquitin–proteasome pathway of degradation (Ref. 80). The only studies performed to date on heart cells have used neonatal rat cardiac fibroblasts, which similarly respond to rH2 relaxin with reduced collagen expression. However, this effect was again observed only after TGF-β1 stimulation (Ref. 64). It seems that, at least in vitro, stimulation of fibroblasts with a pro-fibrotic agent is required in order to demonstrate the anti-fibrotic effects of relaxin. This implies that although relaxin could prove therapeutically useful in conditions associated with high TGF-β1 levels and collagen overexpression, endogenous relaxin may have only minor relevance in the regulation of collagen synthesis in cardiac fibroblasts under normal circumstances.

The ability of relaxin to decrease collagen synthesis in vitro is supported by several studies in rodent models of fibrosis. In bromoethylamine-induced renal fibrosis in rats, relaxin infusion for 28 days decreased the area of interstitial collagen staining in corticomedullary sections by 75% (Ref. 17). This was associated with a decrease in immunoreactive TGF-β1 and macrophage infiltration. Physiologically, these effects combine to preserve the glomerular filtration rate in relaxin-treated rats, resulting in improved renal function compared with untreated animals (Ref. 17). Similar benefits have been observed after relaxin administration in other rodent models of renal (Ref. 81), pulmonary (Refs 16, 79) and hepatic (Ref. 82) fibrosis. Infusion of rH2 relaxin for a period of 14 days also reversed cardiac fibrosis in 12-month-old male *Rlx*$^{-/-}$ mice. Specifically, there was a ~40% reduction in collagen content (inferred from hydroxyproline measurement) in the ventricles (Ref. 64). Transgenic mice overexpressing β_2ARs in the heart develop severe interstitial fibrosis of the left ventricular myocardium (Ref. 83), and reduced collagen content in the ventricles of these animals is also observed after rH2 relaxin treatment (Ref. 64). Unfortunately, no functional assessment of cardiac function was made in this study so it is not clear if relaxin treatment reduces elevated LVEDP and enhanced late-filling flow velocity (A-wave), both of which are defects observed in male *Rlx*$^{-/-}$ mice. Relaxin treatment of neonatal cardiac fibroblasts in the presence of TGF-β1 induced a modest increase in MMP2 activity (but not MMP9), prompting the authors to speculate that the reduction in relative collagen content in the ventricles of 12-month-old mice could be due to stimulation of MMP2 production (Ref. 64). However, there is no direct evidence in vivo that relaxin upregulates expression or activity of MMP2 in the heart, so it is difficult to conclude that the decrease in ventricular collagen is due to activation of MMP2 in older animals.

Histological analyses of porcine reproductive tissues after treatment with porcine relaxin in vivo demonstrated that relaxin decreases the percentage area of collagen compared with the area of amorphous ground substance in these tissues (Refs 84, 85, 86). These data suggest that the effect of relaxin may be to loosen the ECM without decreasing overall collagen content. However, one problem with histological or hydroxyproline assessment of collagen is that there is little insight into the molecular mechanisms of relaxin action on the balance of collagen synthesis and degradation. Furthermore, hydroxyproline analysis cannot provide any information about the effects of relaxin on ECM structure in situ.

The most significant advance in our understanding of the pro-collagenolytic mechanisms of relaxin activity was made recently by Brown and colleagues, who used second-harmonic generation (SHG) imaging to examine collagen turnover induced by relaxin (Ref. 87). The ability to produce SHG signals is a particular

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property of several biological materials including collagen, and offers several advantages over traditional imaging methods (Ref. 88). Their data showed that relaxin decreased the end-to-end length and brightness of individual collagen fibres in Mu89 human melanomas. They suggested that these changes were caused in part by localised cleavage of fibres and by loss of material from their visible ends, which might involve upregulation of MMP synthesis from fibroblasts. However, the equilibrium SHG signal over the 12-day rH2 relaxin infusion period was maintained, indicative of increased de novo collagen production (Ref. 87). Therefore, the molecular mechanisms of relaxin action within the cardiac ECM may in fact involve a balance between cleavage of pre-existing collagen fibres and stimulation of de novo collagen.

MMP activity

Evidence that relaxin induces collagen fibre cleavage through MMP activation is controversial. Although studies using isolated human dermal, lung and lower uterine segment fibroblasts demonstrate dose-dependent increases in MMP1 mRNA expression after treatment with relaxin (Refs 77, 79, 89), this has not been reported in vivo. Relaxin has also been demonstrated to alter TIMP expression in a variety of in vitro and in vivo models (Refs 77, 82, 89, 90), but whether or not relaxin has this effect in the myocardium is unknown. Only one study to date has examined the effects of relaxin on MMP expression in heart tissues in vivo; there were no significant alterations in MMP13 or MMP9 mRNA levels in the atria or ventricles of male $Rlx^{-/-}$ mice (Ref. 59). Cardiac TIMP expression was not examined in this or any other study. As mentioned previously, relaxin increases MMP2 activity measured in culture medium obtained from neonatal rat cardiac fibroblasts (Ref. 64). However, there are no differences in ventricular or atrial MMP2 mRNA levels between 12-month-old $Rlx^{-/-}$ and $Rlx^{+/+}$ mice in vivo (Ref. 59). In summary, there is little available evidence to demonstrate that endogenous relaxin promotes collagen degradation in the heart by increasing MMP expression or activity.

NO biosynthesis

A decade ago, Masini and colleagues suggested that inhibition of histamine release from rat and guinea pig mast cells by relaxin occurred through a NO-dependent mechanism (Ref. 91). Relaxin-induced NO generation or NOS upregulation has since been demonstrated in a variety of tissues and cell types, including vascular smooth muscle cells (Ref. 92), coronary endothelial cells (Ref. 93), MCF-7 breast adenocarcinoma cells (Ref. 94) and gastrointestinal smooth muscle (Ref. 95). Relaxin also induces gelatinase activity in renal vasculature and thereby stimulates NO biosynthesis (Ref. 96). In the heart, NO is thought to mediate the ability of relaxin to increase coronary blood flow (Ref. 97) and to protect against cardiac anaphylaxis (Ref. 98). The potential effects of relaxin on fibroblast differentiation and myofibroblast survival have not been explored, but it is also possible that relaxin stimulates myofibroblast apoptosis through upregulation of the NO biosynthetic pathway. However, endogenous relaxin inhibits cervical epithelial and stromal cell apoptosis from mid-to-late gestation (Refs 99, 100), whereas studies in $LGR7^{-/-}$ and $Rlx^{-/-}$ mice implicate relaxin as an anti-apoptotic factor for immature sperm cells in the testis (Refs 71, 101). These data would seem to contradict the above hypothesis. However, no study to date has examined the effect of relaxin on apoptosis outside the reproductive tract or in (myo)fibroblast cells.

Clinical implications

There are three main areas of focus concerning the potential clinical application of relaxin: heart failure, ischaemia/reperfusion injury and cardiac fibrosis. As described below, it has also recently been suggested that relaxin potentiates cellular cardiomyoplasty (CCM) and might have a protective role against cardiovascular disease.

Heart failure

Dschietzig and colleagues reported that cardiac expression and plasma concentrations of relaxin are higher in patients with moderate and severe CHF (Ref. 60). Furthermore, increases in plasma relaxin were directly correlated with increased left ventricular filling pressure. Relaxin is also thought to be released predominantly into the coronary circulation, because concentrations in the coronary sinus were higher than in the left ventricle in ~80% of CHF patients (Ref. 60). These authors suggested that relaxin may be a compensatory mediator in CHF, counteracting the vasoconstricting and salt-retaining effects of neurohumoral mediators associated with heart

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failure such as AII and endothelin 1 (ET-1). In support of this, human relaxin attenuates both haemodynamic and AII-induced upregulation of ET-1 by increasing the expression of the ET_B receptor, which is thought to remove circulating ET-1. Accordingly, plasma ET-1 and relaxin levels are inversely correlated in severe heart failure (Ref. 60). However, Kerchner and colleagues were unable to replicate the finding that relaxin increases the endothelial expression of ET_B (Ref. 102). It was surmised that stimulation of vasodilation, atrial natriuretic peptide release and matrix degradation could contribute to a compensatory role for relaxin in heart failure. However, other studies failed to show an elevation of relaxin concentrations in CHF patients (Ref. 61), and do not support the concept that relaxin is a player in heart failure (Ref. 62). Furthermore, relaxin concentrations are not useful indicators of prognosis in heart failure patients, unlike pro-B-type natriuretic peptide cleavage products (Ref. 103), so the role of relaxin or its therapeutic use in CHF are still unclear.

Ischaemia/reperfusion injury

Myocardial ischaemia/reperfusion injury resulting from coronary vessel occlusion is associated with myocyte necrosis, endothelial damage and cardiac dysfunction. Both resident cardiac cells and leukocytes that have been recruited to the area initiate secretory programs that result in increased local levels of inflammatory (e.g. histamine) (Ref. 104) and matrix-degrading mediators (e.g. MMPs) (Ref. 105) during the period of ischaemia and reperfusion. In addition, the production of oxygen-derived free radicals exacerbates endothelial and myocardial cell damage initiated by the hypoxic conditions (Ref. 106). Although the effect of ischaemia/reperfusion on the expression of relaxin peptides in the heart is relatively unknown, relaxin has recently been reported to reduce the severity of myocardial injury resulting from ischaemia/reperfusion. Administration of porcine relaxin to rats 30 minutes before ligature of the left anterior descending artery reduced the extent of injured myocardial area and the onset of ventricular arrythmias, thereby decreasing post-ischaemic mortality. These outcomes were associated with decreased mast cell degranulation and reduction in neutrophil accumulation and cell membrane lipid peroxidation, as well as attenuation of calcium overload (Ref. 107). These effects were thought to be mediated primarily by NO stimulation. Although relaxin might attenuate neutrophil extravasation into myocardial tissues directly (Ref. 108), some of these effects are likely to be secondary to preservation of coronary and collateral blood flow through relaxin-induced NO generation and subsequent vasodilation during ischaemia. In support of this concept, treatment of isolated guinea pig hearts with porcine relaxin at the time of occlusion maintains coronary blood flow in association with increased nitrite production (Ref. 109).

Other work has suggested that relaxin might induce angiogenesis in the myocardium, which would additionally increase its cardioprotective effect in ischaemia/reperfusion events (Refs 110, 111). In female Sprague-Dawley rats with left coronary artery occlusion, human relaxin upregulates basic fibroblast growth factor (bFGF) expression at peri-infarct sites, and induces neoangiogenic blood vessel formation. In human fetal cardiac cells in vitro, human relaxin increases bFGF and vascular endothelial growth factor (VEGF) expression in a dose-related manner (Ref. 110). The angiogenic effect of relaxin might also be potentiated by its ability to stimulate ECM degradation, which may be important for neoangiogenic blood vessel formation in the heart (Ref. 112). Through the dual effects of vasodilation and angiogenesis, relaxin could effectively enhance collateral circulation in ischaemic myocardium, thereby exerting a significant cardioprotective effect. Relaxin administration could therefore constitute a novel therapeutic approach in the treatment of myocardial ischaemia/reperfusion injury.

Cardiac fibrosis

Although there is some evidence that relaxin reduces collagen expression in vitro, it seems premature at present to assign a therapeutic use for relaxin in the treatment of heart conditions associated with fibrosis, as there are almost no data on the regulation of myocardial collagen by relaxin. Furthermore, the supposed anti-fibrotic effects of relaxin do not seem as clear-cut as previously suggested (Ref. 64). Although relaxin could degrade fibrillar collagen in the heart in accordance with its 'classical' actions, it might also upregulate de novo ECM synthesis (Ref. 87). Therefore, the predominant effects of relaxin on collagen and ECM metabolism in post-MI ventricular fibrosis, for example, are difficult to

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predict. Moreover, it is unclear if post-MI ECM-targeting therapies should aim to modulate the balance in favour of deposition or degradation. A better understanding of the temporal and spatial (i.e. infarct versus non-infarct tissue) considerations of post-MI collagen remodelling is required before effective intervention in the clinical setting will be possible (Ref. 33).

Other clinical implications

An exciting development revealed in recent investigations is the use of relaxin to potentiate CCM. This novel cellular transplantation technique, often of autologous skeletal myoblasts, has been used for the regeneration of ventricular myocardium after infarction (Ref. 113). In vitro, relaxin improves intercellular coupling between rat cardiomyocytes and mouse skeletal myoblasts by increasing gap junction formation and transcellular conductance, suggesting that relaxin could enhance the functional integration of skeletal myoblasts into infarcted tissue and therefore the success of CCM in vivo (Ref. 113). This group of researchers have also hypothesised, in light of the epidemiological evidence of a low incidence of cardiac disease in menstruating women and the lack of a clear benefit of post-menopausal steroid supplementation to cardiovascular illness, that circulating relaxin might afford fertile women protection against ischaemic heart disease (Ref. 114). The authors speculate that relaxin or relaxin-derived drugs could be used to prevent or treat cardiac ischaemia by exerting vasodilatory and anti-platelet aggregatory actions (Ref. 114). Moreover, relaxin increases the NO-synthesising capability of the vasculature by upregulating the synthesis of iNOS (Ref. 93) and promotes structural remodelling of vessel walls (Ref. 115), thereby increasing vasodilatory capacity and promoting vascular compliance. Therefore, relaxin might be an effective anti-hypertensive agent through its ability to moderate chronic cardiovascular tone.

Research in progress and outstanding research questions

Relaxin ligand and receptor expression

There is still no consensus as to which relaxin peptides and receptors are expressed in the heart or the degree of variation among species. Techniques used in previous studies that excluded the presence of relaxin receptors from the ventricles may not have been sufficiently sensitive to detect low receptor expression in this tissue (Ref. 67). Relaxin ligand and receptor expression must be examined quantitatively in the heart in vivo to identify the relevant components of the cardiac relaxin system. Furthermore, circulating relaxin concentrations reported in heart failure patients show a high degree of variability among research groups using the same relaxin ELISA (Refs 60, 61, 62), and clarification of this issue would be helpful. The detection limit of the human relaxin ELISA used in these studies was reported to be as low as 0.4 pg/ml (Ref. 60), which is tenfold more sensitive than the human relaxin ELISA used in earlier studies (Refs 116, 117, 118). The validity of the latter ELISA has been demonstrated with data on recoveries after spiking plasma with known amounts of rH2 relaxin (Refs 116, 117, 118). To our knowledge, no validation data have been published for the more sensitive assay, so comparisons between studies are difficult. Further study is also needed to clarify whether or not physiological or pathological stimuli directly alter cardiac expression of relaxin peptides and receptors.

Collagen degradation

Although there is some evidence to indicate that relaxin reduces total collagen in the heart, the ability of relaxin to induce collagen degradation in vivo has yet to be established. Moreover, the mechanisms by which it might do so are almost completely unknown. Relaxin-stimulated upregulation of cardiac MMP expression or activity has largely been demonstrated in vitro, and these data are difficult to interpret given the complex aetiology (including age and/or disease) often associated with cardiac fibrosis in vivo. However, Jeyabalan et al. (Ref. 119) recently demonstrated that relaxin infusion in non-pregnant rats resulted in increased MMP2 protein in small renal arteries. These data support a role for relaxin in the regulation of vascular gelatinase expression that may also have relevance for the actions of relaxin in the heart. Another possibility is that relaxin reduces de novo collagen production, which may account for the decrease in cardiac collagen content in response to relaxin treatment (Ref. 64), but this has not been demonstrated in rodent models of cardiovascular disease. Adding to the controversy is a lack of functional data, so it is not known how relaxin-stimulated decreases in cardiac collagen might translate into improved cardiac

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performance. This makes it difficult to speculate on the clinical significance of relaxin-induced changes in the ECM. Thus, functional assessment must be made where possible to assess the therapeutic effects of relaxin in animal models of cardiac fibrosis.

Recent studies of collagen turnover in tumours (Ref. 87) suggest that relaxin not only increases collagen fibre degradation, but also upregulates de novo collagen synthesis. That relaxin apparently induces both collagen synthesis as well as activation of factors involved in collagen degradation perhaps suggests that extracellular fragmentation of collagen could stimulate de novo collagen production in fibroblasts. This hypothesis assumes that relaxin does not have a direct effect on stimulating collagen synthesis. Collagen propeptide fragments have previously been shown to induce collagen production in fibroblast cultures (Ref. 120), so delineating the precise effects of relaxin (both direct and indirect) will be an important area for future research in ECM regulation in the heart.

Concluding remarks

In conclusion, factors that influence the balance of ECM synthesis and degradation in the myocardium are likely to be useful therapeutic targets in specific clinical settings such as cardiac ischaemia/reperfusion injury. In vitro studies suggest that, in cardiac conditions associated with ECM overexpression and fibrosis, relaxin could assist in the degradation of excess interstitial collagen, leading to reversal of fibrosis and increased myocardial compliance. Several in vivo studies conducted in models of renal and hepatic fibrosis support this concept, and there is some evidence to suggest that relaxin may reduce the total collagen content of the heart. However, there is currently a lack of data concerning the molecular actions of relaxin in the cardiac ECM in vivo. It is possible that relaxin stimulates both collagen degradation and (via a positive-feedback loop) collagen synthesis in the heart, which might attenuate the anti-fibrotic potential of relaxin. Relaxin treatment might nonetheless exert some acute functional benefit on fibrotic myocardium despite stimulating de novo collagen synthesis, as newly synthesised collagen does not possess the structural rigidity of mature collagen. Moreover, novel anti-fibrotic actions of relaxin, such as attenuation of fibroblast differentiation and upregulation of the urokinase-type plasminogen activator/plasmin system or myofibroblast apoptosis could be additional mechanisms by which this peptide alleviates fibrosis in the heart.

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Further reading, resources and contacts

'The Relaxin Newsletter: a Resource for Relaxinologists' is an electronic publication co-edited by an international group of relaxin researchers. Seven issues have been published to date. E-mail relaxin_news@hotmail.com to subscribe.

Information and program details of the Third and Fourth Conferences on Relaxin and Related Peptides, held in Australia and USA in October 2000 and September 2004, respectively, can be found at:

http://www.hfi.unimelb.edu.au/relaxin2000 and http://www.life.uiuc.edu/relaxin2004

BAS Medical acquired the worldwide rights to recombinant human relaxin in 2003. Information on product development and potential indications for relaxin currently under investigation can be found at:
http://www.basmedical.com

Features associated with this article

Figures

Figure 1. A generalised scheme of cardiac fibrosis.
Figure 2. A schematic representation of prorelaxin.

Table

Table 1. A comparison of the human relaxins.

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