

REVIEW ARTICLE

The actions of relaxin on the human cardiovascular system

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The insulin-like peptide relaxin, originally identified as a hormone of pregnancy, is now known to exert a range of pleiotropic effects including vasodilatory, anti-fibrotic, angiogenic, anti-apoptotic and anti-inflammatory effects in both males and females. Relaxin produces these effects by binding to a cognate receptor RXFP1 and activating a variety of signalling pathways including cAMP, cGMP and MAPKs as well as by altering gene expression of TGF- β , MMPs, angiogenic growth factors and endothelin receptors. The peptide has been shown to be effective in halting or reversing many of the adverse effects including fibrosis in animal models of cardiovascular disease including ischaemia/reperfusion injury, myocardial infarction, hypertensive heart disease and cardiomyopathy. Relaxin given to humans is safe and produces favourable haemodynamic changes. Serelaxin, the recombinant form of relaxin, is now in extended phase III clinical trials for the treatment of acute heart failure. Previous clinical studies indicated that a 48 h infusion of relaxin improved 180 day mortality, yet the mechanism underlying this effect is not clear. This article provides an overview of the cellular mechanism of effects of relaxin and summarizes its beneficial actions in animal models and in the clinic. We also hypothesize potential mechanisms for the clinical efficacy of relaxin, identify current knowledge gaps and suggest new ways in which relaxin could be useful therapeutically.

LINKED ARTICLES

This article is part of a themed section on Recent Progress in the Understanding of Relaxin Family Peptides and their Receptors. To view the other articles in this section visit <http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.10/issuetoc>

Abbreviations

ADHF, acute decompensated heart failure; AHF, acute heart failure; Ang II, angiotensin II; AC, arterial compliance; b-FGF, basic FGF; BMDECs, bone marrow-derived endothelial cells; CI, cardiac index; CO, cardiac output; ET-1, endothelin-1; ECM, extracellular matrix; iCa^{2+} , intracellular calcium; IR, ischaemia-reperfusion; mRen2, murine renin gene; MI, myocardial infarction; PGF, placental growth factor; PVR, pulmonary vascular resistance; SHR, spontaneously hypertensive rat; SVR, systemic vascular resistance; TIMP, tissue inhibitors of metalloproteinase; TGF- β 1, transforming growth factor β 1; VSMC, vascular smooth muscle cells; VAS, visual analogue scale

Tables of Links

TARGETS	
Enzymes^a	PI3K
ACE	PKA
Adenylyl cyclase	PKC- ζ
Akt	GPCRs^b
Connexins	AT ₂ receptor
eNOS	ET _B receptors
ERK1/2	RXFP1 receptors
Guanylyl cyclase	Nuclear hormone receptors^c
iNOS	Glucocorticoid receptors
nNOS	

LIGANDS	
Ang II, angiotensin II	NO
cAMP	PD123319
CCL2 (MCP-1)	Relaxin
cGMP	SU5416 (semaxanib)
Enalapril	TGF- β 1
ET-1, endothelin-1	TIMP-1
Indomethacin	TNF- α
Isoprenaline	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (^{a,b,c}Alexander *et al.*, 2015a,b,c).

Introduction

Relaxin is a heterodimeric peptide hormone closely related structurally to insulin. The major circulating form of relaxin is that produced by the *RLN2* gene and is the cognate ligand for the RXFP1 receptor, a classical GPCR containing seven transmembrane spanning regions as well as a large extracellular domain with 10 leucine-rich repeats and a unique N-terminal LDL receptor type A module (Hsu *et al.*, 2002). Relaxin is closely related to insulin-like peptide 3, relaxin-3 and insulin-like peptide 5, which are the cognate ligands for the relaxin family peptide receptors RXFP2, RXFP3 and RXFP4 respectively (Alexander *et al.*, 2015b). *RXFP1* mRNA and protein are found in mammalian reproductive tissues but also in the heart, arteries, kidney, lung, liver and blood cells as well as in a number of areas of the brain (Dschiertzig *et al.*, 2001b; Bathgate *et al.*, 2013; Halls *et al.*, 2015). It is now recognized that relaxin acting through RXFP1 receptors has pleiotropic effects including vasodilation, anti-fibrotic, angiogenic, anti-apoptotic and anti-inflammatory effects (Figure 1).

The pleiotropic effects of relaxin

Relaxin, originally identified as a hormone of pregnancy, mediates many of the cardiovascular and renal adaptations of pregnancy (Conrad and Shroff, 2011). Relaxin-neutralizing antibodies or ovariectomy prevents the rise in arterial compliance (AC) and cardiac output (CO) and the fall in systemic vascular resistance (SVR) seen in pregnant rats (Debrah *et al.*, 2006) and prevents the renal vasodilation normally observed during pregnancy (Novak *et al.*, 2001). These effects are also observed in non-pregnant animals (Danielson *et al.*, 1999; Conrad and Novak, 2004; Debrah *et al.*, 2005a).

Other chronic effects of relaxin include important and consistent inhibitory effects on stimulated extracellular matrix (ECM) deposition. It inhibits proliferation and differentiation of fibroblasts into myofibroblasts (activated fibroblasts

that promote ECM/collagen deposition and fibrosis) in rat cardiac (Samuel *et al.*, 2004a), renal (Masterson *et al.*, 2004), hepatic (Williams *et al.*, 2001) and human pulmonary tissues (Unemori *et al.*, 1996; Huang *et al.*, 2001); it also activates various ECM/collagen-degrading MMPs and inhibits tissue inhibitors of metalloproteinase (TIMP) activity (Samuel *et al.*, 2007; Bennett, 2009). Additionally, the peptide represents an important driver of VEGF-related angiogenesis and wound healing (Unemori *et al.*, 1992; Unemori *et al.*, 2000).

Relaxin has anti-apoptotic and anti-hypertrophic effects in human trophoblasts (Lodhi *et al.*, 2013), human osteosarcomas (Ma *et al.*, 2013a), epithelial and stromal cells (Lee *et al.*, 2005) and rodent cardiomyocytes (Moore *et al.*, 2007; Samuel *et al.*, 2011). These anti-apoptotic effects of relaxin are dependent on RXFP1 receptors as the peptide has no apoptotic effects in RXFP1^{-/-} mice (Yao *et al.*, 2009).

Relaxin has anti-inflammatory effects in humans and animals. It inhibits vascular inflammation by inhibiting TNF- α -induced expression of vascular cell adhesion molecule, platelet endothelial cell adhesion molecule and the chemokine CCL2 (Brecht *et al.*, 2011). It furthermore inhibits markers of inflammation in an NO-dependent manner by attenuating activation of neutrophils (Bani *et al.*, 2002; Masini *et al.*, 2004) and basophils (Bani *et al.*, 2002) and by inhibiting histamine release from mast cells (Masini *et al.*, 1995). Relaxin also inhibits the aggregation of isolated human and rabbit platelets (Bani *et al.*, 1995a) and decreases the number of circulating platelets in rats.

Cellular basis for the actions of relaxin

Signal transduction pathways activated by relaxin

cAMP cAMP is involved in many physiological responses including inotropy and chronotropy in the heart and vasodilation in the vasculature (Houslay *et al.*, 2007). Acute administration of relaxin increases cAMP levels in HEK-293,

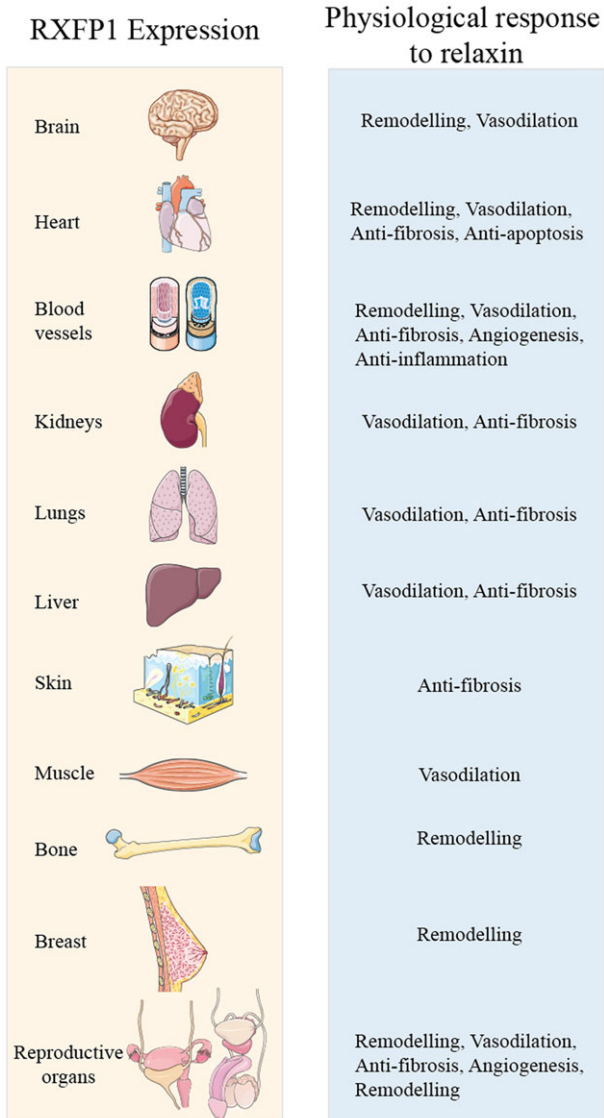


Figure 1

The tissue distribution of RXFP1 receptors and the physiology of relaxin in humans. Relaxin has vasodilatory, anti-fibrotic and remodelling effects in various reproductive and non-reproductive organs.

THP-1 and MCF-7 cells; rat renal and cardiac fibroblasts; rat heart; human endothelial and smooth muscle cells; and fibroblasts from human arteries and veins (Kompa *et al.*, 2002; Nguyen and Dessauer, 2005a; Halls *et al.*, 2009; Horton *et al.*, 2011). The characteristics of relaxin-mediated cAMP accumulation are dependent on the cellular background. In HEK-RXFP1 cells, there is increased cAMP accumulation associated with coupling to G_{α_s} modulated by $G_{\alpha_{OB}}$ and a delayed increase in cAMP mediated through $G_{\alpha_{13}}$ (Halls *et al.*, 2006), $\beta\gamma$ subunits, PI3K and translocation of PKC- ζ to the cell membrane to activate AC (Nguyen and Dessauer, 2005b; Halls *et al.*, 2006). The $G_{\alpha_{13}}$ - $G\beta\gamma$ -PI3K-PKC- ζ pathway also operates in THP-1 cells, in human endothelial and smooth muscle cells and in rat and human cardiac fibroblasts but is missing in rat renal fibroblasts and Colo16 cells, where *Pertussis* toxin

pretreatment enhances relaxin-mediated cAMP accumulation (Halls *et al.*, 2009).

NO and cGMP Relaxin utilizes NO to produce vasodilation *in vitro* (Bani-Sacchi *et al.*, 1995; Bani *et al.*, 1998a; McGuane *et al.*, 2011b) and *in vivo* (Jeyabalan *et al.*, 2003; Conrad and Shroff, 2011). *In vitro*, relaxin rapidly activates the NO/cGMP pathway in vascular smooth muscle, coronary artery endothelium, bovine aortic smooth muscle, human breast cancer cells, perfused guinea pig and rat hearts and mouse uterus (Bani-Sacchi *et al.*, 1995; Bani *et al.*, 1995b; Bani *et al.*, 1998a,b; Failli *et al.*, 2002; Masini *et al.*, 2002) as well as rat cardiac (Samuel *et al.*, 2004a) and renal (Mookerjee *et al.*, 2009; Chow *et al.*, 2014) fibroblasts. This is achieved by increasing the activity and/or expression of NOS. Relaxin interacts with all three NOS subtypes: endothelial NOS (eNOS) (McGuane *et al.*, 2011b), inducible NOS (iNOS) (Failli *et al.*, 2002; Quattrone *et al.*, 2004) and neuronal NOS (nNOS) (Chow *et al.*, 2012; Mookerjee *et al.*, 2009), to increase NO production, although the interaction is not well understood and is highly species- and tissue-dependent. Vasodilatory responses and phosphorylation of eNOS in human coronary artery endothelial cells to acutely administered relaxin are attenuated by inhibition of $G_{\alpha_{i/o}}$ coupling to PI3K, PKB/Akt, indicating that NO/cGMP production is dependent on $G_{\alpha_{i/o}}$, PI3K, Akt and NOS (Figure 2) (McGuane *et al.*, 2011b). However, in rat renal fibroblasts, chronic relaxin over 72 h promotes nNOS-derived NO/cGMP signalling through G_{α_s} and $G_{\alpha_{OB}}$

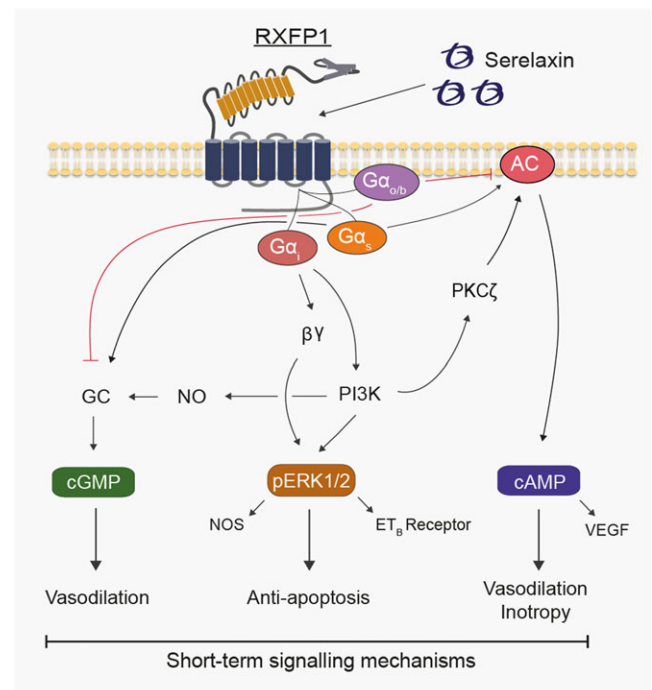


Figure 2

Mechanisms involved in short-term (minutes) relaxin-mediated signal transduction in human primary vascular cells.

(Mookerjee *et al.*, 2009; Chow *et al.*, 2014). *In vivo*, the NOS/NO/cGMP pathway is critical for relaxin-induced vasodilation and cardioprotection. Relaxin protects rat lungs after ischaemia-reperfusion (IR) injury through iNOS induction (Alexiou *et al.*, 2013), reduces infarct size in rat brains by eNOS activation (Wilson *et al.*, 2006) and reduces cardiac infarct size in the pig through a NOS-dependent mechanism (Masini *et al.*, 1997) but also reduces fibrosis when given chronically in a bleomycin-induced lung fibrosis mouse model through an NO/cGMP-dependent pathway (Huang *et al.*, 2011).

MAPKs. The MAPK ERK1/2 inhibits apoptosis and promotes cell survival and is likely to play key roles in cardiomyocyte survival in heart failure (HF) (Bueno and Molkentin, 2002). Relaxin stimulation causes a transient increase in ERK1/2 phosphorylation (pERK1/2) in HEK-RXFP1 cells, THP-1 cells (Nguyen and Dessauer, 2005a), HeLa cells (Dschietzig *et al.*, 2003), human endometrial stromal cells (Zhang *et al.*, 2002), rat renal fibroblasts (Chow *et al.*, 2012), human smooth muscle cells (Zhang *et al.*, 2002) and fibrocartilaginous cells (Ahmad *et al.*, 2012). A more prolonged pERK1/2 response occurs in HUVECs and immortalised HUVECs (Eahy926 cells) (Dschietzig *et al.*, 2003; Dschietzig *et al.*, 2009a). Relaxin rapidly increases pERK1/2 in human vascular cells in a G_{α_i} - and PI3K-dependent manner (Sarwar *et al.*, 2015; Figure 2), and this likely mediates the anti-apoptotic and cardiac cell survival effects of relaxin in humans. Other MAPKs such as p38MAPK are also activated, but this response is dependent on the cellular background. Thus, relaxin increases p38MAPK in human vascular smooth muscle cells (VSMC) but not in HUVEC or HeLa cells (Dschietzig *et al.*, 2003), whereas in human renal fibroblasts, relaxin does not affect pERK1/2, p38MAPK or pJNK (Heeg *et al.*, 2005).

Calcium. Relaxin has no direct effect on basal intracellular calcium (Ca^{2+}) levels but inhibits agonist (angiotensin II (Ang II) and thrombin)-mediated Ca^{2+} in rat VSMC and endothelial cells, rat coronary artery endothelial cells, bovine aortic VSMC and guinea pig hearts (Bani *et al.*, 1998b; Failli *et al.*, 2002; Masini *et al.*, 2002), perhaps reflecting that RXFP1 receptors are not G_{α_q} coupled. Although G_{α_i} coupling increases Ca^{2+} (Kiselyov *et al.*, 2003), this does not occur with the relaxin/RXFP1 receptor system, as relaxin does not affect Ca^{2+} signalling. Relaxin most likely acts as a physiological antagonist by producing vasodilator effects that counteract the vasoconstrictor actions of Ang II and endothelin-1 (ET-1) that cause Ca^{2+} release via their respective receptors.

Chronic changes produced by relaxin

Matrix metalloproteinases. The anti-fibrotic effects observed with chronically administered relaxin include inhibition of transforming growth factor $\beta 1$ (TGF- $\beta 1$), promotion of the expression and/or activity of ECM/collagen-degrading MMPs and inhibition of TIMP activity (Samuel *et al.*, 2007). *In vitro* relaxin increases MMP-2 activity in human renal (Heeg *et al.*, 2005) and rat cardiac (Samuel *et al.*, 2004a; Heeg *et al.*, 2005) and renal (Chow *et al.*, 2014) fibroblasts, whereas it increases MMP-9 activity in THP-1 cells, rat renal fibroblasts and

temporomandibular joint (TMJ) cells (Heeg *et al.*, 2005; Ho *et al.*, 2007; Ahmad *et al.*, 2012; Chow *et al.*, 2014). Relaxin-induced activation of MMP-2, MMP-9 and MMP-13 in human and rat renal fibroblasts is associated with iNOS-induced NO production (Chow *et al.*, 2012) and inhibition of Smad-2 phosphorylation and thus TGF- $\beta 1$ activation (Heeg *et al.*, 2005; Mookerjee *et al.*, 2009). Relaxin also interacts with other MMPs in a tissue-dependent manner because in TMJ fibrocartilaginous cells, relaxin increases MMP-1, MMP-9 and MMP-13 activity but only increases MMP-13 activity in rat ventricular fibroblasts (Kapila *et al.*, 2009; Hossain *et al.*, 2011; Ahmad *et al.*, 2012). In human dermal fibroblasts and rat renal myofibroblasts, relaxin increased MMP-2 and MMP-9 by a mechanism involving RXFP1 receptors, pERK1/2 and NO/cGMP (Figures 2 and 3) (Chow *et al.*, 2012). Similarly, in fibrocartilaginous cells, relaxin increased MMP-9 and MMP-13 by stimulating RXFP1 receptors and activating PI3K, Akt, ERK and PKC- ζ and the transcription factors Elk-1 and c-fos (Ahmad *et al.*, 2012). NF- κB also has a role in relaxin-mediated MMP-9 activation in human THP-1 and fibrocartilaginous cells (Ho *et al.*, 2007; Ahmad *et al.*, 2012).

Angiogenic growth factors. Involvement of growth factors and cytokines in the angiogenic effects of chronic relaxin is conflicting and likely to be tissue and cell specific. In THP-1 cells, relaxin increases the expression of VEGF (VEGF₁₆₅ and VEGF₁₂₁) and basic FGF (b-FGF) and causes angiogenesis at ischaemic wound sites in rodents (Unemori *et al.*, 2000). Relaxin stimulates VEGF production in human endometrial cells (Unemori *et al.*, 1999), Saos-2 cells (Ma *et al.*, 2013b), THP-1 cells (Unemori *et al.*, 2000) and human endothelial cells (Unemori *et al.*, 1992). The mechanism involves cAMP in normal human endometrial cells where relaxin induces VEGF expression in a cAMP-dependent manner without affecting b-FGF, TGF- $\beta 1$ and placental growth factor (PGF) (Figures 2 and 3) (Unemori *et al.*, 1999). Although VEGF receptor inhibitors prevent relaxin-induced increases in GFR and decreases in renal vascular resistance without affecting mean arterial pressure (McGuane *et al.*, 2011a), in rat small renal arteries, SU5416 increases relaxin-mediated vasodilation (McGuane *et al.*, 2011b). These apparently contradictory effects may relate to actions of SU5416 other than VEGF receptor blockade that include inhibition of the tyrosine kinases FLT-3 and RET and other protein kinases such as c-Kit and Met (Mologni *et al.*, 2006). However, VEGF and PGF neutralization prevents relaxin-mediated inhibition of myogenic constriction in mouse and rat small renal and human subcutaneous arteries (McGuane *et al.*, 2011a), suggesting that VEGF and PGF may have a role in relaxin-mediated vasodilation.

In mice, relaxin increases circulating levels of bone marrow-derived endothelial cells (BMDECs) that facilitate angiogenesis and contribute to repair of vascular endothelium. BMDECs are recruited to the site of angiogenesis where relaxin promotes vasculogenesis by RXFP1 receptor activation, leading to NO generation and PI3K and Akt signalling (Segal *et al.*, 2012; Ma *et al.*, 2013b). This may explain the healing properties of relaxin because chronic administration in diabetic mice increases VEGF levels in the wound area and promotes skin recovery, an effect reversed by administration of VEGF antibodies (Bitto *et al.*, 2013).

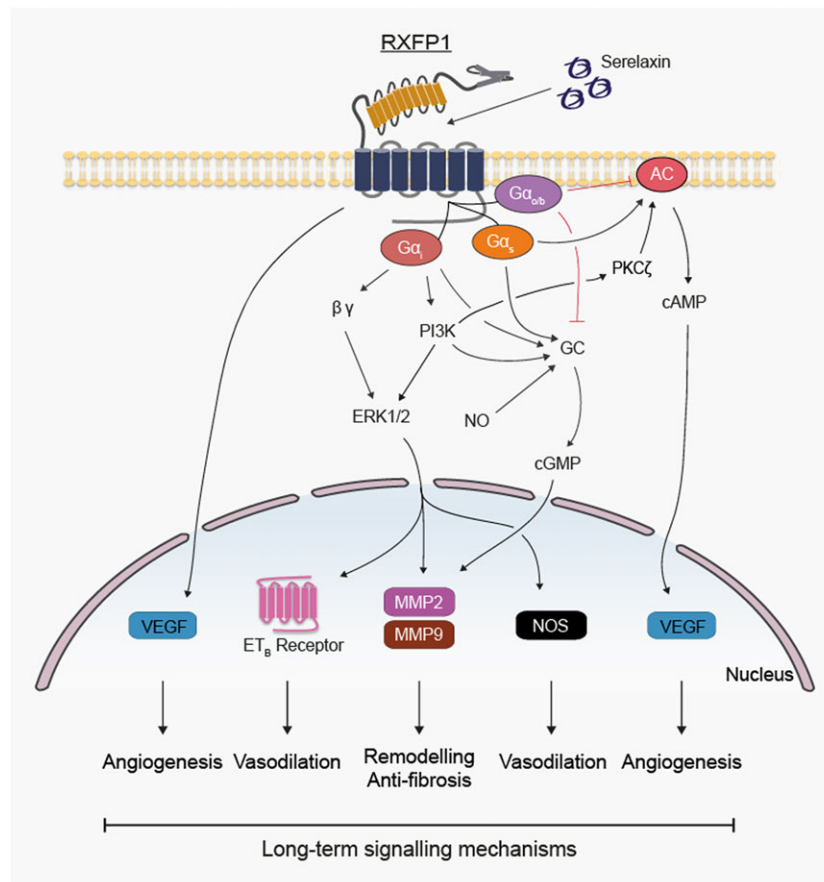


Figure 3

Effects of longer-term (hours to days) relaxin administration on expression of genes likely to influence signal transduction in human primary vascular cells.

Endothelin receptors. Endothelin receptors are secondary mediators in the vasodilatory responses to relaxin. In HeLa cells, HUVECs and bovine aortic endothelial cells, but not in human VSMC, relaxin increases mRNA and protein of the ET_B receptor by a Ras-independent Raf-1–MEK-1–ERK1/2 kinase cascade that activates NF- κ B (Figures 2 and 3), and the vasoconstrictor responses to ET-1 and ET-3 in rat aorta strips are inhibited by relaxin treatment in an ERK1/2- and ET_B -dependent manner (Dschietzig *et al.*, 2003). The role of ET_B receptors in vasodilatory responses to relaxin is strongly affected by cell and tissue background. In small renal arteries from virgin, midterm pregnant and non-pregnant rats, relaxin has no effect on ET_B receptor levels (Kerchner *et al.*, 2005). In lung IR injury, relaxin is protective, but the effect does not involve ET_B receptors (Alexiou *et al.*, 2013). However, in human coronary artery endothelial cells and HUVECs but not in human aortic endothelial cells or human dermal microvascular endothelial cells, relaxin produced small and inconsistent increases expression of ET_B receptors (Kerchner *et al.*, 2005). This apparent discrepancy was explained by the use of small rather than large arteries, no pulmonary endothelial cells and the significantly lower relaxin concentrations used in the latter study. Furthermore, small renal arteries from relaxin-treated non-pregnant rats showed reduced myogenic reactivity, an

effect not observed in mice lacking ET_B receptors (Jeyabalan *et al.*, 2003). More recent studies with 24 and 48 h exposure to relaxin (1.68 nM) show modest but consistent increases in ET_B receptors in HUVEC, human umbilical arterial smooth muscle cells and human cardiac fibroblasts and marked increases in human umbilical venous smooth muscle cells (Sarwar *et al.*, 2015).

Actions of relaxin in the cardiovascular system

Relaxin targets vascular cells

There is evidence from preclinical and clinical studies that relaxin targets the vasculature. In rats, chronic relaxin increases CO, global AC, renal plasma flow and GFR and reduces SVR and myogenic constriction without affecting mean arterial pressure (Danielson *et al.*, 1999; Conrad *et al.*, 2004; Debrah *et al.*, 2005a). Given acutely, relaxin specifically vasodilates rat mesenteric arteries, rodent small renal and mesenteric arteries (McGuane *et al.*, 2011b) and human small gluteal and subcutaneous arteries but not pulmonary resistance arteries (McGuane *et al.*, 2011b). Relatively little is known about the effects of relaxin on veins despite relaxant effects on

mesenteric veins without affecting myogenic reactivity (Li *et al.*, 2005) and powerful effects on signalling in human umbilical venous smooth muscle cells (Sarwar *et al.*, 2015). The regionally specific effects of relaxin can be explained by differential RXFP1 receptor expression in different vascular beds. Thus, in rats, RXFP1 receptors are highly expressed in endothelial cells of the aorta, vena cava, mesenteric artery and vein as well as in VSMC of the femoral artery and vein and small pulmonary arteries (Jelinic *et al.*, 2014).

Signal transduction pathways activated by relaxin in vascular cells

Relaxin given acutely causes cAMP, cGMP and pERK1/2 signalling in endothelial cells, VSMC and fibroblasts from human arteries and veins (Sarwar *et al.*, 2015), mediated by G_{α_s} , $G_{\alpha_{i/o}}$ and $G_{\alpha_{OB}}$ G-proteins and involving PI3K downstream of G-protein activation (Figure 2). These pathways likely mediate the rapid dilation observed in human subcutaneous arteries and rodent renal and mesenteric arteries where acute relaxin rapidly activates G_{α_i} , PI3K and NOS signalling (McGuane *et al.*, 2011b). Longer-term relaxin also changes gene expression in endothelial cells, VSMC and fibroblasts from human arteries and veins with increased gelatinases (MMP-2 and MMP-9), ET_B receptors, VEGF and NOS in most but not all cell types (Sarwar *et al.*, 2015) (Figure 3). These changes may account for the haemodynamic changes observed during pregnancy and *in vivo*, particularly the increase in AC and decrease in SVR (thereby improving CO) observed in pregnant (Debrah *et al.*, 2006) and non-pregnant animals (Conrad *et al.*, 2004). More importantly, these changes are likely to account for the widespread haemodynamic changes observed in patients with acute HF (AHF) treated with serelaxin (Ponikowski *et al.*, 2014).

Relaxin promotes vascular cell crosstalk

The endothelium secretes vasoactive substances including NO, PGs and endothelium-derived hyperpolarizing factor that regulate smooth muscle tone. Responses to relaxin were abolished in human gluteal arteries denuded of endothelium (Fisher, 2009), and in an *in vitro* co-culture model, the relaxin responses in VSMC were endothelium dependent and governed by expression of RXFP1 receptors in endothelial cells (Figure 4) (Sarwar *et al.*, 2016).

NO synthesis regulates relaxin-mediated crosstalk between endothelial cells and VSMC in the human vasculature (Figure 4). This is observed *in vitro* in human arterial/venous endothelial cells and VSMC (Sarwar *et al.*, 2016), *in vivo* in conscious rats where NO inhibition abolished relaxin-mediated increases in renal vasodilation (Danielson *et al.*, 1999) and in rat and guinea pig hearts where NO inhibition abolished relaxin-mediated increases in coronary flow (Bani-Sacchi *et al.*, 1995).

Prostanoids are also involved in specific circumstances. In human coronary artery endothelial cells but not HUVEC, relaxin promotes COX activity to cause an increase in cAMP accumulation in co-cultured smooth muscle cells (Sarwar *et al.*, 2016). In *ex vivo* human gluteal arteries, indomethacin (a non-specific COX inhibitor) almost abolished relaxin-mediated vasodilation (Fisher, 2009), suggesting that, in

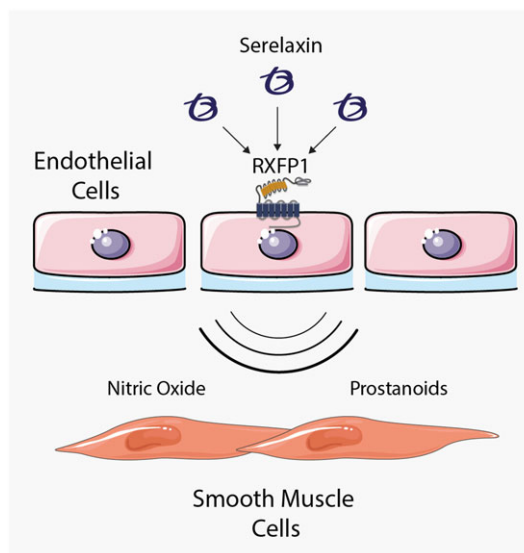


Figure 4

Relaxin only activates endothelial cells that express cell surface RXFP1 receptors to promote cellular crosstalk between endothelial cells and smooth muscle cells involving NO and, in some endothelial cells, prostanoids.

some circumstances, there is a role for prostanoids in relaxin signal transduction.

Actions of relaxin on the heart

The mammalian heart contains relaxin binding sites that are exclusively found in the atrium (Osheroff *et al.*, 1992; Tan *et al.*, 1999). Acutely administered relaxin has positive chronotropic and inotropic effects in the rat isolated perfused heart and isolated atria, with a potency greater than ET-1, Ang II or isoprenaline (Kakouris *et al.*, 1992; Coulson *et al.*, 1996; Toth *et al.*, 1996).

Relaxin inhibits injury-induced hypertrophy and apoptosis in neonatal rat cardiomyocytes and increases the growth and maturation of neonatal mouse cardiomyocytes. RXFP1 receptors are expressed in cardiac fibroblasts from several species including rodents and humans where they cause short-term and long-term changes in signal transduction (Sarwar *et al.*, 2016) (Figures 2 and 3). Relaxin also acts on the pacemaker cells of the rabbit sino-atrial node by modulating L-type Ca^{2+} current to increase heart rate. The effect of relaxin was not observed when administered following the β -adrenoceptor agonist isoprenaline or the cell permeable cAMP analogue 8-Br-cAMP that produced similar effects. The responses to relaxin, isoprenaline and 8-Br-cAMP were blocked by the PKA inhibitor PKI, suggesting that they were all mediated by a cAMP/PKA-dependent pathway (Han *et al.*, 1994).

Effects of relaxin in human tissues

Human blood vessels

In general, studies on human vessels are in concordance with mechanisms elucidated in rodents (McGuane *et al.*,

2011a,b). Relaxin has both acute and longer-term vasodilatory properties. Acutely (within minutes), relaxin dilates arteries by activating eNOS (Bani *et al.*, 1998a; McGuane *et al.*, 2011b; Dschietzig *et al.*, 2012), PI3K, Akt and PKA with subsequent eNOS phosphorylation at Ser¹¹⁹⁵, Ser⁶¹³ and Thr⁴⁹⁵ and NO generation (McGuane *et al.*, 2011b; Dschietzig *et al.*, 2012). Over a longer period (h), the ET_B receptor mediates NO-dependent vasodilation (Fernandez-Patron *et al.*, 1999; Dschietzig *et al.*, 2003). In mesenteric and renal resistance vessels of ~100–300 µm diameter, relaxin activates MMP-9 and later MMP-2 to generate the ET-1 peptide, ET₁₋₃₂ from its precursor, big ET-1 (Jeyabalan *et al.*, 2003). ET₁₋₃₂, though a more potent vasoconstrictor than ET₁₋₂₁ in endothelium-denuded rat mesenteric arteries, preferentially interacts with the endothelial ET_B receptors due to its site of generation in intact vessels (Fernandez-Patron *et al.*, 2000) and precipitates the release of NO (Fernandez-Patron *et al.*, 1999; McGuane *et al.*, 2011b). VEGF and PGF are also involved in sustained vasodilation, but the precise mechanism involved is still obscure; the VEGF and PGF effects appear to be located downstream of MMPs and initiated by MMP-induced release of matrix-bound angiogenic factors (McGuane *et al.*, 2011b).

An alternative mechanism in larger vessels and the pulmonary circulation is relaxin-induced up-regulation of endothelial ET_B receptors (see above), which expression of RXFP1 receptors mediates NO production and, equally importantly, ET-1 clearance (Dschietzig *et al.*, 2001a,b; Dschietzig *et al.*, 2003). Finally, relaxin promotes angiogenesis both in humans and in rodents (Unemori *et al.*, 1999; Unemori *et al.*, 2000) by inducing the synthesis of VEGF and b-FGF. This may serve to improve tissue and organ perfusion in the long term.

Human heart

In contrast to the evidence in rodents, little information is available regarding human myocardial tissue. Immunohistochemical studies (Dschietzig *et al.*, 2001b) of left ventricle from patients with end-stage HF indicate relaxin expression in interstitial cells and cardiomyocytes. The expression of RXFP1 receptors has been described in human cardiac fibroblasts (Sarwar *et al.*, 2015) and cardiomyocytes. In human atrial and ventricular samples obtained from donor and terminally failing explanted hearts (Dschietzig *et al.*, 2011), relaxin addition evoked a potent inotropic effect in atrial (EC₅₀ below 1 nM), but not ventricular, myocardium from both normal and failing hearts. This effect critically involves PKA and inhibition of the rapidly inactivating potassium outward current, *I_{to}*. Additionally, the contribution of Gα₁₃-PI3K signalling clearly increases in failing human atria, in accord with Gα₁₀ up-regulation in chronic HF (CHF) (Eschenhagen *et al.*, 1992). RXFP1 receptors were expressed in atrial, but not ventricular, myocardium from both control and failing hearts.

Many questions remain regarding the role of the relaxin system in human myocardium including whether there is expression of RXFP1 receptors in adult ventricular cardiomyocytes and which of the canonical and non-canonical signalling pathways, apart from Gα₁₃-PI3K, are active and how they are regulated.

Haemodynamic effects of relaxin in the cardiovascular system

Haemodynamic effects *in vivo*: vascular actions

Relaxin appears to have a facilitatory role in cardiovascular adaptation during pregnancy but is not essential at least in the mouse since a majority of relaxin-1 knockout (KO) mice produce litters (Zhao *et al.*, 1999). Reduction in SVR in pregnant women is partially attributable to the vasodilatory action of elevated circulatory levels of relaxin (Sherwood, 2004). In rodents, acute or chronic relaxin induces vasodilation, reduced BP, a decline in arterial stiffness, reduction in SVR and an increase in CO and renal blood flow (Conrad and Novak, 2004). Haemodynamic changes seen during pregnancy and with relaxin administration are similar. There is a sustained reduction of vascular resistance following a bolus injection of relaxin, involving bradykinin- and prostacyclin-mediated vasodilatation (Leo *et al.*, 2014b). Relaxin (up to 6 h) reduced SVR and increased CO in hypertensive rats chronically infused with Ang II, while in an spontaneously hypertensive rat (SHR) model, similar haemodynamic effects were evident only after relaxin therapy for several days (Debrah *et al.*, 2005b). Whereas increased CO was not reported by other studies, chronic relaxin therapy for 2 weeks reduced large artery stiffness by lowering vascular collagen content in aged SHR (Xu *et al.*, 2010). Collectively, these pre-clinical findings indicate that several mechanisms are responsible for the reduction in vascular resistance following acute and chronic relaxin therapy (Du *et al.*, 2014).

Haemodynamic effects *in vivo*: cardiac actions

In humans, unlike rodents, administration of relaxin even at high doses did not increase heart rate (Ponikowski *et al.*, 2014), perhaps reflecting lower expression of RXFP1 receptors in human heart, relative to rodents. The inotropic action of relaxin involves both Gα_s modulated by Gα_{OB} (Kompa *et al.*, 2002), which also facilitates PKA signalling by activation of the Gα₁₃-PI3K pathway (Halls *et al.*, 2015). In isolated myofilaments, relaxin activates PKC/Ca²⁺ signalling, leading to positive inotropy (Shaw *et al.*, 2009). In failing and non-failing human atria, relaxin increased contractile force, by signalling that is PKA-dependent and negatively regulated by Gα_i (Dschietzig *et al.*, 2011). There is no evidence for a ventricular inotropic action of relaxin in rodents *in vivo* (Lekgabe *et al.*, 2005; Xu *et al.*, 2010; Samuel *et al.*, 2011). Collectively, studies in atrial tissues from rodents have convincingly shown chronotropic and inotropic actions of relaxin. In humans, the cardiac index (CI) was unchanged by relaxin treatment although afterload was reduced (Ponikowski *et al.*, 2014) and, apparently, relaxin does not produce inotropy in ventricular myocardium nor increase heart rate in human hearts.

Studies in models lacking RXFP1 receptors or relaxin (KO models)

The male but not the female relaxin KO mouse displays widespread organ fibrosis involving the reproductive system, nipples, skin, lungs, heart and kidney, with treatment with relaxin for up to 2 weeks able to reverse established fibrosis in many organs and tissues, including the heart (Zhao *et al.*,

2000; Du *et al.*, 2003; Samuel *et al.*, 2003; Samuel *et al.*, 2004a, b). Relaxin influences vascular proliferation in the endometrium, is associated with increased regional blood flow during pregnancy and promotes uterine and placental growth. These adaptive changes are significantly attenuated in relaxin-1 KO mice and reversed by relaxin treatment (Gooi *et al.*, 2013). Male relaxin KO mice display reduced compliance of resistance arteries (renal, uterus, mesentery and femoral arteries) and an attenuated vasodilatory response, implying that endogenous relaxin is required to maintain normal vascular structure and function (Gooi *et al.*, 2013; Leo *et al.*, 2014a; Jelinic *et al.*, 2015; Ng *et al.*, 2015).

RXFP1 receptors comprise the major type of relaxin receptor in the cardiovascular system (Bathgate *et al.*, 2013; Halls *et al.*, 2015). In RXFP1 receptor KO mice, relaxin-induced increases in NO production by endothelial cells were abolished (Segal *et al.*, 2012). No reduction in tissue collagen content and remodelling of the reproductive tract was seen in conditional RXFP1 receptor KO mice, a phenotype similar to that of relaxin KO mice (Krajnc-Franken *et al.*, 2004; Kaftanovskaya *et al.*, 2015).

Relaxin in animal models of cardiovascular disease

IR and myocardial infarction (MI)

Following cardiac IR, the injured myocardium displays sequential cell death, microvascular damage, innate immune response and subsequent reparative and/or reactive myocardial fibrosis which is limited by relaxin. In guinea pigs, rats and pigs, reduction in myocardial injury by relaxin administered prior to reperfusion was associated with attenuated Ca^{2+} overload, oxidative stress, histamine release or inflammatory cell infiltration (Masini *et al.*, 1997; Bani *et al.*, 1998b; Formigli *et al.*, 2007). In the porcine IR model, treatment with relaxin or regional delivery of myoblasts transfected with the relaxin gene improved viability of engrafted myoblasts and improved healing (Formigli *et al.*, 2005; Formigli *et al.*, 2007). Interestingly, the onset of ventricular arrhythmias during acute IR was reduced in relaxin-treated rat or porcine hearts, likely to be due to reduced myocardial injury (Bani *et al.*, 1998b; Nistri *et al.*, 2008). *In vitro* studies show that relaxin protects against cardiomyocyte apoptotic death induced by oxidative stress (Moore *et al.*, 2007), hypoxia-reoxygenation (Boccalini *et al.*, 2015) or high glucose exposure (Zhang *et al.*, 2015) and involved activation of Akt together with increased Bcl-2/Bax ratio, Notch-1 activation or suppressed endoplasmic reticulum (ER) stress.

In myocardial ischaemia, microvascular damage associated with regional inflammation and regional leukocyte interaction with vascular endothelial cells represents a pivotal part of overall cardiac injury (Kloner, 2011). In cultured endothelial cells, exposure to cytokines, inflammatory signalling and leukocyte adhesion were attenuated by relaxin (Brecht *et al.*, 2011). In the pig IR model (Formigli *et al.*, 2007), the anti-fibrotic effect was associated with a reduction in infarct size, while in the mouse MI model (without reperfusion) (Samuel *et al.*, 2011), infarct size was unchanged, and yet fibrotic healing was attenuated by relaxin without adverse

influence on the extent of post-infarct ventricular remodelling and function.

Hypertensive heart disease

The effects of relaxin have been studied in rodent models of hypertension such as SHR, drug-induced renal injury, transgenic expression of murine renin gene (mRen2) plus diabetes or hypertension induced by chronic infusion of Ang II. In SHR or rats with Ang II-induced hypertension, relaxin therapy (1–7 days) reduced pulmonary vascular resistance (PVR) and vascular stiffness and increased renal blood flow and CO, leading to unchanged mean BP (Debrah *et al.*, 2005b) with the response to relaxin being quicker in Ang II than in SHR models. In SHR rats with left ventricular hypertrophy, endogenous myocardial relaxin mRNA and protein levels were higher than in age-matched Wistar control rats and correlated inversely with left atrial and left ventricular mass index (Dschiezic *et al.*, 2005). While this was supported by *in vitro* findings showing inhibition of cardiomyocyte hypertrophy induced by a fibroblast-conditioned medium (Moore *et al.*, 2007), other *in vivo* studies on elderly SHR did not show changes in heart weight following chronic relaxin treatment for 2 weeks (Lekgabe *et al.*, 2005; Xu *et al.*, 2010).

Studies in 9-month-old SHRs provided strong evidence for anti-fibrotic effects of relaxin, and treatment for 2 weeks (at $500 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) effectively reduced cardiac and renal fibrosis (Lekgabe *et al.*, 2005). Reduced atrial fibrosis following 2 week relaxin therapy (Parikh *et al.*, 2013) and, in 17-month-old SHRs, improved large artery compliance, measured *in vitro* and *in vivo*, together with a significant reduction in arterial collagen content were observed (Xu *et al.*, 2010).

In the salt-sensitive Dahl rat, relaxin attenuated the rise in BP and increased renal nNOS and eNOS (Yoshida *et al.*, 2011), and selective inhibition of the three NOS isoforms blocked the anti-hypertensive effects of relaxin. Relaxin for 6 weeks significantly decreased systolic BP, glomerular and tubulointerstitial changes and TGF- β signalling. Relaxin also ameliorated renal ischaemic reperfusion injury, improved renal function and reduced circulating and renal expression of TNF- α (Yoshida *et al.*, 2013). In rats with renal injury caused by bromoethylamine or reduced renal mass, relaxin for 4 weeks preserved renal function, improved renal creatinine clearance and ameliorated proteinuria, oxidative stress and renal fibrosis, which occur together with a reduction or absence of renal hypertension (Garber *et al.*, 2001; Garber *et al.*, 2003). Ang II infusion into rats caused hypertension, albuminuria, glomerular sclerosis and enhanced oxidative stress, which were improved by relaxin for 2-weeks. These effects were abolished by L-NAME, indicating an NO-dependent mechanism (Sasser *et al.*, 2011).

Chronic relaxin was ineffective in rats with dual transgenic expression of mRen2 and angiotensinogen, early onset of hypertension, rapid development of organ damage (cardiomyocyte hypertrophy, loss of glomeruli, fibrosis, renal damage and severe diastolic dysfunction with preserved systolic function) and early premature death (over 70% by week 7) (Haase *et al.*, 2014). This severe and rapidly developing model makes testing therapeutic efficacy difficult, and in addition, the plasma levels of relaxin in this model may have been suboptimal. Considering the reciprocal effects of cardiac-renal injury or dysfunction (Braam *et al.*, 2014), further research is warranted

to simultaneously investigate effects of relaxin on cardiac and renal injury in animal models of disease.

Cardiomyopathy

Preclinical studies in rodents have used cardiomyopathy induced by chronic treatment with the β -adrenoceptor agonist isoprenaline or transgenic overexpression of human β_2 -adrenoceptors (Bathgate *et al.*, 2008; Hossain *et al.*, 2011; Samuel *et al.*, 2014). In the isoprenaline model in rats, relaxin administered with or after isoprenaline administration protected hearts against injury measured by levels of circulating biomarkers and decreased cardiac fibrosis (Zhang *et al.*, 2005; Samuel *et al.*, 2014). Comparison of the anti-fibrotic efficacy of relaxin and the ACE inhibitor enalapril in mice with cardiac fibrosis induced by isoprenaline showed that co-administered relaxin or enalapril for 17 days effectively reduced cardiac fibrosis with relaxin displaying better efficacy. Delayed treatment with relaxin was also effective.

In mRen2 hypertensive rats with streptozotocin-induced diabetes, relaxin therapy for 2 weeks reduced cardiac fibrosis and suppressed fibroblast proliferation and TIMP-1 activity (Samuel *et al.*, 2008). In mice with type 2 diabetes by high-fat diet feeding for 13 weeks, relaxin administration over the last 3 weeks reduced fasting glucose level, improved insulin sensitivity, restored endothelial-dependent vasodilatation and reduced cardiac and hepatic fibrosis (Bonner *et al.*, 2013).

Clinical studies with relaxin

CHF and AHF

The first dose-finding trial in patients with stable systolic CHF (Dschiertzig *et al.*, 2009b) indicated that relaxin infusion ($10\text{--}960 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) for up to 24 h was safe and evoked favourable haemodynamic changes. Thus, it elevated CI without affecting heart rate; decreased pulmonary capillary wedge pressure, SVR and PVR; and did not significantly alter systolic BP/mean BP or right atrial pressure. Haemodynamic changes generally started within 45–60 min. Pharmacokinetics in stable patients with systolic CHF and impaired renal function (Voors *et al.*, 2014) showed that relaxin ($30 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ over 24 h) did not change systolic BP compared with placebo. In AHF patients, who usually present with systolic BP higher than in stable CHF patients (Gheorghiane *et al.*, 2006), systolic BP was decreased by relaxin with patients with higher initial BP experiencing larger effects (Teerlink *et al.*, 2013; Voors *et al.*, 2011). This suggests a self-limiting effect on BP – advantageous for organ protection during AHF (Voors *et al.*, 2011).

In a haemodynamic study in AHF patients (Ponikowski *et al.*, 2014), the changes with relaxin ($30 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) confirmed the findings of the pilot trial in stable CHF (Dschiertzig *et al.*, 2009b), with the significant exception of CI being unchanged. One explanation may be that the high adrenergic drive usually seen in dyspnoeic AHF patients would subside as soon as pulmonary congestion is relieved by relaxin, so introducing a confounder regarding CI. In addition, in AHF patients, urine output and fluid balance are not controlled, and as these are improved by relaxin, the more negative volume balance may have affected CI. The Pre-RELAX-AHF trial (Teerlink *et al.*, 2009) identified the optimal dose of serelaxin,

$30 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, and confirmed the safety of the drug as well as several trends towards beneficial outcomes.

To date, the phase III RELAX-AHF trial involving more than 1100 patients is the largest cardiovascular relaxin trial (Teerlink *et al.*, 2013). Major inclusion criteria included dyspnoea at rest or on minimal exertion, pulmonary congestion on chest radiograph, elevated levels of B-type natriuretic peptides, mild to moderate renal dysfunction, systolic BP exceeding 125 mmHg, enrolment within 16 h of hospital admission and administration of ≥ 40 mg of furosemide before enrolment. Patients with acute coronary syndrome were excluded, and dose reductions of relaxin upon excessive systolic BP drops were pre-specified. The primary end points were dyspnoea relief measured as improvement on visual analogue scale (VAS) on day 5, as well as on the Likert scale after 6, 12, and 24 h of therapy. The secondary end points included mortality and re-hospitalization rates within 60 days. Among the tertiary end points, 180-day mortality and different safety assessments were pre-specified. Of note, mean systolic BP at baseline was 142 mmHg, higher than in any previous AHF study, ~40% of all patients had atrial fibrillation, and ~45% showed an ejection fraction $>40\%$. The mean time to inclusion was uniquely short (8 h). RELAX met one primary end point, the improvement on VAS, but failed to meet the second, related to the Likert scale. All secondary end points showed a neutral outcome. Multiple, statistically unadjusted, comparisons indicated less administration of intravenous diuretics; less worsening of renal function and worsening of HF before day 14; and less dyspnoea, oedema and rales in the rhRlx group. The effects of relaxin were not dependent on certain subgroups of the study population (Metra *et al.*, 2013b).

A major and rather unexpected finding was the relaxin-related benefit in terms of 180 day mortality, with an improvement from 9.6 to 6.1% for cardiovascular and from 11.3 to 7.3% for all-cause mortality and a number needed to treat of approximately 25. The safety analysis showed a significant decrease in systolic BP at different time points (by 4–6 mmHg), more frequent dose modifications (29 vs. 18%), but similar rates of hypotensive adverse events and no systolic BP rebound after drug cessation in the serelaxin, as compared with the placebo, group. Of note, a combined analysis of the Pre-RELAX and RELAX trials (Teerlink *et al.*, 2009; Teerlink *et al.*, 2013) suggests that relaxin primarily reduced deaths classified as ‘non-HF cardiovascular cause’ and ‘sudden death’, though this conclusion is merely hypothesis generating, because of the overall small number of events (Greene and Gheorghiane, 2014). As RELAX-AHF was not powered to detect mortality differences, a second and larger mortality trial in AHF patients is underway.

The encouraging results obtained in RELAX were also corroborated in the (much smaller) Japanese Serelaxin trial (Sato *et al.*, 2015) underlining the efficacy of the peptide in an Asian population.

Current knowledge gaps

Mechanism of 48 h treatments having effects after 180 days

Certainly, the study design of RELAX considered the lessons from the many failed AHF studies by enrolling earlier after

AHF onset and by focusing exclusively on patients with rather high BP. But what are the critical roles of relaxin in reversing or reducing damage associated with HF? The rather moderate and self-limiting effect on BP should be considered because it is most likely to prevent renal adverse events (Gheorghiadu *et al.*, 2006; Voors *et al.*, 2011).

With regard to signal transduction, it is worth mentioning the inhibitory effect of relaxin on ET-1 that has a pivotal role in AHF-related systemic and pulmonary afterload mismatch, neurohumoral activation and inflammation (Webb, 1995; Stangl *et al.*, 2000; Kawanabe and Nauli, 2011). Relaxin inhibits the stimulation of ET-1 release, reduces ET-1 gene expression, promotes endothelial expression of its clearance receptor, ET_B, and acts thereby as a functional ET-1 antagonist (Dschietzig *et al.*, 2001a,b; Dschietzig *et al.*, 2003). Also, activation of the cGMP-PKG pathway is highly beneficial in many pathological cardiovascular states (Paulus and Tschope, 2013). Relaxin feeds into this pathway at many points, by stimulating NO production and/or NOS expression (Bani *et al.*, 1998a; Alexiou *et al.*, 2013) and by increasing NO bioavailability through its anti-inflammatory (Masini *et al.*, 2004; Brecht *et al.*, 2011), antioxidant and endothelium-protective actions (Dschietzig *et al.*, 2012).

The anti-fibrotic effects of relaxin may play a role in organ protection, and reversal of established cardiac fibrosis by relaxin is desirable particularly in patients with HF with preserved ejection fraction (Figure 5). The peptide also exerts a broad range of vascular and organ-protective (Alexiou *et al.*, 2013; Collino *et al.*, 2013) actions mediated mainly via NO and the glucocorticoid receptors (Dschietzig *et al.*, 2004; Dschietzig *et al.*, 2012). Numerous *in vitro* and *in vivo* studies show cardiac protection following brief administration of relaxin against acute injury due to ischaemia, oxidative stress, inflammation or drug toxicity (Du *et al.*, 2014). That these factors precipitate cardiac decompensation or acute worsening of HF has been well established (Cotter *et al.*, 2010; Januzzi *et al.*, 2012; Metra *et al.*, 2013a). Thus, the cardiac protective property of relaxin might result in suppression of reactive and/or reparative fibrosis (Figure 5).

A key finding in the RELAX-AHF trial is that the dynamic changes in the first couple of days of biomarkers that reflect damage of multiple organs (heart, kidney and liver) and have predictive value for 180 day prognosis in patients with acute decompensated HF (ADHF) are reduced by relaxin (Metra *et al.*, 2013a). In fact, in addition to these pivotal organs, there is also evidence for damage or malfunction of other organs such as intestine, lung and skeletal muscle, in the setting of ADHF (Peschel *et al.*, 2003; Lauten *et al.*, 2011; Hogan *et al.*, 2012a,b). Thus, we suggest that a compromised tissue microcirculation underlies the multiple organ damage or malfunction (Du *et al.*, 2014). Considering the present strong evidence for organ and endothelial protection by relaxin in a variety of disease settings (Du *et al.*, 2014), we find it likely that damage and dysfunction of the microvasculature in many organs in response to acute decompensation of HF are also exacerbated in clinical conditions following ADHF. In this context, acute relaxin administration protects against microvascular injury, poor microcirculation and organ damage. The amelioration of organ damage and dysfunction by short-term administration of relaxin would affect long-term prognosis by limitation of cardiovascular injury and reduction of reparative and reactive fibrosis (Figure 5).

Role of venodilation by relaxin in treating AHF

The effects of relaxin on the venous circulation could potentially account for the haemodynamic effects of relaxin in AHF and CHF. One recent study in rat mesenteric veins showed that, in contrast to arteries, they are not remodelled and do not show reduced myogenic reactivity in response to relaxin (Li *et al.*, 2005; Jelinic *et al.*, 2014). Both studies were performed *in vitro* and may have been influenced by venous tone being significantly more dependent than arterial tone on sympatho-adrenergic innervation and pacemaker cells (Cajal cells) (Gelman, 2008). Contraction of splanchnic veins by relaxin (as seen in pregnancy; Slangen *et al.*, 1996; Edouard *et al.*, 1998) or dilation of central veins would increase venous return and explain the baseline-dependent, 'self-limiting' drop of BP observed in AHF and CHF patients treated with

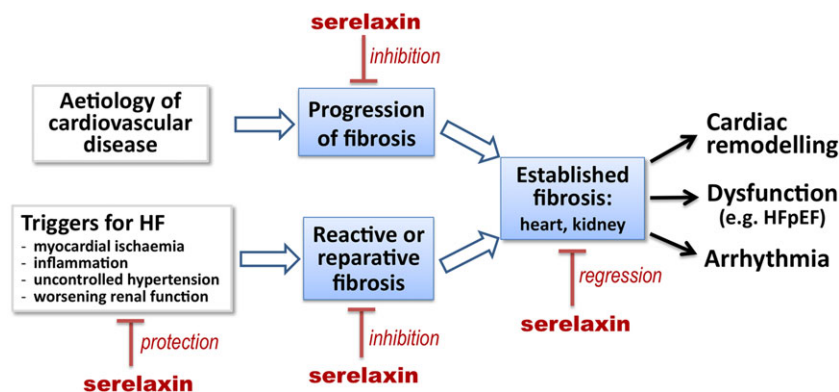


Figure 5

Therapeutic potential of serelaxin in heart disease and HF. Relaxin therapy mediates fibrosis reversal in rodent models of cardiac fibrosis. There is also strong experimental evidence and clinical evidence for the cardiac protective property of relaxin against factors that function as triggers for cardiac decompensation or acute worsening of HF. These actions would be expected to ameliorate cardiac fibrosis.

relaxin. The issue is therefore still open for interesting and important investigations.

Anti-arrhythmic potential

Reduction of 180 day mortality by relaxin treatment was observed in the RELAX-AHF trial (Teerlink *et al.*, 2013). With some of the sudden deaths likely to be attributable to arrhythmias (Felker *et al.*, 2014), there is emerging evidence for an anti-arrhythmic action of relaxin. In an elderly SHR model (Parikh *et al.*, 2013), relaxin ($0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) over 2 weeks followed by programmed electrical stimulation of the isolated atria reduced atrial fibrillation, an effect associated with significant reduction in atrial fibrosis and increased Na^+ current density (Parikh *et al.*, 2013). It has been suggested that there is a reciprocal influence between HF and arrhythmias and that arrhythmias act as a facilitating factor for decompensation or worsening of HF (Dukes *et al.*, 2015; Kotecha and Piccini, 2015). Fibrosis reversal and anti-arrhythmic effects are observed after relaxin for 14 days, although it is not known if this could be achieved by a 48 h treatment. Further studies are necessary to explore the time course of anti-fibrotic and potentially anti-arrhythmic actions by relaxin either as a monotherapy or in conjunction with other cardiac medications.

There is also evidence that suggests that relaxin influences myoendothelial coupling in humans. Gap junctions play an important role in vessel structure, function and therefore myoendothelial coupling. Gap junctions are composed of connexin (Cx) proteins, and in the cardiovascular system, the main connexins expressed are Cx40, Cx37, Cx43 and Cx45 (de Wit *et al.*, 2008). Relaxin increases the expression of Cx43 in several tissues, including mouse neonatal immature cardiomyocytes, rodent cardiomyocytes and skeletal myoblasts (Formigli *et al.*, 2005) and hearts from SHR (Parikh *et al.*, 2013). There is also a role of Cx proteins in cellular crosstalk because relaxin promotes the coupling between mouse skeletal muscle myoblasts and adult rat ventricular cardiomyocytes in an *in vitro* co-culture model that involves Cx43, suggesting a potential role for gap junction communication that is enhanced by relaxin (Formigli *et al.*, 2005). Thus, there is a need to thoroughly investigate the effects of relaxin on myoendothelial coupling to better understand its cardiovascular effects in humans.

Regulation of RXFP1 receptors or of relaxin in the cardiovascular system under diseased conditions

Endogenous expression of relaxin is elevated in heart disease (Dschiezig *et al.*, 2001b; Xu *et al.*, 2008) although the extent of up-regulation seems insufficient to exert compensatory action (Xu *et al.*, 2008). Regulation of relaxin expression is poorly defined. The rat relaxin gene promoter region contains guanine (G)-rich repeats, allowing formation of G-quadruplexes that can be stabilized by ligands such as berberine (Gu *et al.*, 2012). These sequences are adjacent to the binding motif of STAT3 that negatively regulates relaxin expression. In cardiac fibroblasts and in rats treated with Ang II, berberine suppressed fibroblast activation, collagen synthesis and cardiac fibrosis by up-regulating relaxin (Gu *et al.*, 2012).

There is limited information on gene regulation of RXFP1 receptors in disease. The expression of RXFP1 receptors is moderately decreased in failing human myocardium (Dschiezig *et al.*, 2011). In reproductive tissues, expression of RXFP1 receptors is up-regulated by oestrogen and progesterone (Yan *et al.*, 2008; Vodstrcil *et al.*, 2010), but oestrogen treatment has no effect or may even reduce expression in atria (Tan *et al.*, 1999). It remains unknown whether expression of RXFP1 receptors in the cardiovascular system is gender-dependent or varies during pregnancy. While mRNA for RXFP1 receptors was up-regulated in the infarcted rat heart (Kompa *et al.*, 2002), expression of these receptors was unchanged in a mouse model of chronic pressure overload (Xu *et al.*, 2008). The expression of RXFP1 receptors in cultured cardiomyocytes or intact heart is down-regulated by activation of β_1 -adrenoceptors but up-regulated by activation of α_1 -AR (Moore *et al.*, 2014). This may influence the efficacy of serelaxin in patients treated with β -blockers. This was not observed in the RELAX-AHF trial (Metra *et al.*, 2013a), perhaps due to small sample size. This question, however, could be addressed in the current RELAX-AHF2 trial.

Could the standard therapy given to patients in RELAX-AHF be confounding the results, that is, could ACE inhibitors/AT receptor antagonists/ β -blockers affect the response to relaxin?

The pleiotropic actions of relaxin make it unique in AHF therapy. Cardiac remodelling is fundamental in heart disease and failure, and several key factors emerge regardless of aetiology including hypertrophy, inflammation, interstitial fibrosis and continuous loss of cardiomyocytes. These components orchestrate functional decline and development of complications, thereby influencing short- and long-term prognosis of AHF. In theory, changes in some of these key components would be expected to have a beneficial influence on patients with AHF and thus confound the effects of relaxin. On the other hand, ACE inhibitors, Ang II receptor antagonists and β -blockers have not been shown to be effective in AHF.

The evidence that RXFP1 receptors form heterodimers with the Ang II AT_2 receptor and that these are essential for the anti-fibrotic effects of relaxin in the kidney (Chow *et al.*, 2014) raises important questions that are relevant to its clinical use. The anti-fibrotic effects of relaxin are completely lost in mice treated with the AT_2 receptor antagonist PD123319 or in AT_2 receptor KO mice. These findings provide an explanation for the observation that the anti-fibrotic effects of relaxin are only seen in pathological conditions, with no effect on normal tissue. AT_2 receptors are typically expressed at very low levels in healthy tissue, but their expression dramatically increases after injury or disease (Matsubara, 1998; Carey, 2005). RXFP1 receptors are also poorly expressed at the cell surface, but at higher levels within the ER (Kern *et al.*, 2007). Enhanced heterodimerization facilitated by the injury-induced increase in AT_2 receptor expression may increase trafficking of RXFP1 receptors (as an AT_2 –RXFP1 receptor heterodimer) to the cell surface, thus explaining why relaxin treatment is more effective under pathological conditions. While the AT_2 receptor is not currently regarded as a therapeutic target, there is emerging evidence that the RXFP1 receptor forms heterodimers with other GPCRs that are therapeutically important. It is also

interesting to note that, in an earlier study, arteries obtained from patients on ACE inhibitors showed marked attenuation of the vasodilator response to relaxin, effects that appeared to be further enhanced by inhibition of COX (Fisher *et al.*, 2002). However, patients not receiving ACE inhibitors showed little effect of inhibition of COX on the response to relaxin (Fisher *et al.*, 2002).

Conclusions

After a long gestation period, the peptide hormone relaxin is now emerging as an effective treatment for AHF. Although a great deal is now known regarding the cellular actions of relaxin, it is still far from clear which of its pleiotropic effects – vasodilation, anti-fibrotic, angiogenic, anti-apoptotic or anti-inflammatory – is responsible for the beneficial effects. This review has summarized current knowledge of the cardiovascular effects of relaxin, identified knowledge gaps and has suggested possible mechanisms of action.

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Conflict of interest

The authors declare no conflicts of interest.

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