CHAPTER 6

The Vascular Actions of Relaxin

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Abstract

Relaxin is emerging as a hormone with important vascular actions. Much of our recently gained knowledge of relaxin in this context has stemmed from investigations of maternal vascular adaptations to pregnancy in which the hormone is turning out to be an important mediator. This chapter is separated into three parts. In Part 1, we discuss relaxin in the setting of normal vascular function and focus on systemic hemodynamics and arterial mechanical properties, renal and other peripheral circulations, angiogenesis, as well as the cellular mechanisms of the vasodilatory actions of relaxin. In this section, we also summarize the evidence for an arterial-derived relaxin ligand-receptor system. In Part 2, we present relaxin in the context of vascular dysfunction and the implications for relaxin as a therapeutic agent in renal and cardiac diseases, ischemia and reperfusion injury, pulmonary hypertension, vascular inflammation and preeclampsia. Finally, in Part 3, we highlight some of the controversies and unresolved issues, as well as suggest a general direction for future relaxin research that is urgently needed.

Introduction

The discovery that the vasculature may be another target of relaxin was made by Frederick L. Hisaw and colleagues. They reported that following administration of relaxin (Rlx) to castrated monkeys, there were profound morphological alterations in the endothelial cells of the endometrium consistent with hypertrophy and hyperplasia, as well as enlargement of arterioles and capillaries.[1,2] The concept that relaxin alters vascular structure and function has been subsequently bolstered by numerous investigations, particularly in the last decade. Much of our recent understanding of relaxin as a vascular hormone has arisen from studies of maternal renal and cardiovascular adaptations to pregnancy in which relaxin is emerging as a pivotal player. The objective of this chapter is to review the vascular actions of relaxin.

Part 1. Contribution of Relaxin to Normal Vascular Function (Table 1)

Influence of Relaxin on Systemic Hemodynamics and Arterial Mechanical Properties

Definitions

Systemic arterial load is defined as the mechanical opposition to the flow of blood out of the left ventricle.[3] There are 2 components. The first is the steady arterial load commonly known as systemic vascular resistance (SVR), which is calculated by the quotient of mean arterial pressure and cardiac output (CO) and results mainly from arteriolar properties. The second is pulsatile arterial

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Table 1. Summary of the contribution of relaxin to normal vascular function

I.	**Systemic Hemodynamics and Arterial Mechanical Properties**
	• RLX administration increases cardiac output and arterial compliance, and reduces systemic vascular resistance and myogenic reactivity in rats.
	• Endogenous, circulating relaxin mediates the increased cardiac output and arterial compliance, and the reduced systemic vascular resistance and myogenic reactivity during mid-term pregnancy in rats.
II.	**Renal Circulation**
	• RLX administration reduces renal vascular resistance and increases renal plasma flow and glomerular filtration rate in rats and humans.
	• Endogenous, circulating relaxin mediates the reduced renal vascular resistance and increased renal plasma flow and glomerular filtration rate during pregnancy in rats.
	• Endogenous, circulating relaxin contributes to increased glomerular filtration rate during pregnancy in women.
III.	**Other Organ Circulations**
	• RLX administration increases blood flow in other organ circulations: coronary, uterus, mammary gland, liver, mesentery and mesocaecum.
IV.	**Angiogenesis**
	• RLX has been shown to be angiogenic in the endometrium, and in the setting of wound healing and myocardial infarction.
V.	**Local Vascular Relaxin Ligand-Receptor System**
	• Recent evidence indicates the local expression of relaxin ligand and receptor in arteries that increases arterial compliance and reduces myogenic reactivity.

load, which becomes relevant because of the inherently pulsatile nature of the cardiac pump and is determined by vessel geometry and wall visco-elasticity, the branching of the vasculature that yields wave propagations and reflections and the mechanical attributes of blood. Together, the steady and pulsatile arterial loads constitute a comprehensive characterization of total hydraulic load in terms of arterial mechanical properties. Global arterial compliance (global AC) is one measure of pulsatile arterial load, which is derived from CO and the diastolic decay of the aortic pressure waveform or more simply from the ratio of the stroke volume and pulse pressure.

Systemic Hemodynamics and Arterial Mechanical Properties during Pregnancy

A fundamental cardiovascular adaptation to human pregnancy is the increase in global AC which reaches a peak by the end of the first or beginning of the second trimester just as SVR reaches a nadir.[4] At least in theory, the increase in global AC is essential to the maintenance of cardiovascular homeostasis during pregnancy for several reasons: (i) by preventing an excessive fall in diastolic pressure which otherwise would decline to precariously low levels due to the large drop in SVR; (ii) by minimizing the pulsatile or oscillatory work (i.e., wasted energy) which otherwise would rise disproportionately to the increase in total work required of and expended by the heart during pregnancy; (iii) by contributing to arterial underfilling along with the reduction in SVR (albeit to a lesser degree than SVR), both of which abet renal sodium and water retention and plasma volume expansion during early pregnancy; and (iv) by preserving steady shear relative to steady shear stress at the blood-endothelial interface in the setting of the hyperdynamic circulation of pregnancy, thus favoring nitric oxide production by the endothelium over that of superoxide and other potentially damaging reactive oxygen species.

Influence of Relaxin Administration on Systemic Hemodynamics and Arterial Mechanical Properties in Nonpregnant Females and Males

Because relaxin mediates the maternal renal circulatory changes during pregnancy in rats,[5] it was logical to consider whether the hormone might also underlie the broader cardiovascular ad-

aptations to pregnancy, i.e., the elevations in CO and global AC, as well as the decline in SVR. To begin addressing this question, Conrad and coworkers first took a pharmacological approach and investigated whether recombinant human relaxin (rhRLX) has the potential to modify systemic cardiovascular function by chronically administering the hormone to nonpregnant female rats.[6] A methodology was developed to measure global AC in conscious, unrestrained rats. rhRLX was infused subcutaneously for 10 days and circulating levels were similar to those seen in rats during early to midterm pregnancy. Significant increases in CO, global AC, as well as reductions in SVR were noted by the earliest time point of measurement (day 2 or 3) and they were maintained throughout the 10 days of rhRLX infusion, the overall magnitude of these changes being ~ 20%. However, mean arterial pressure was unchanged throughout the 10-day hormone infusion, because the fall in SVR was matched by a compensatory increase in CO, the latter mainly due to augmentation of stroke volume. Finally, small renal arteries dissected from female rats after 5 days of rhRLX administration and subsequently mounted in a pressure arteriograph and treated with papaverine and EGTA to block smooth muscle function, exhibited significant increases in compliance in comparison to those arteries isolated from vehicle treated animals.[6] Recent evidence indicates that, in addition to large arteries, small arteries also make an important contribution to the global AC.[7] These results suggest that, in addition to the decrease in vascular smooth muscle tone due to the vasodilatory attribute of relaxin, the increase in global AC observed in vivo was also a consequence of alterations in vascular structure, i.e., cellular components or extracellular matrix in the blood vessel wall. However, the precise nature of the changes in vascular structure contributing to the increase in arterial compliance during rhRLX administration to nonpregnant rats remains to be determined.

Chronic administration of rhRLX over days to either normotensive control or hypertensive, male and female rats mimicked the alterations in systemic hemodynamic and arterial mechanical properties of gestation.[6,8,9] In contrast, the short-term administration of rhRLX over hours was only effective in the angiotensin-II model of hypertension, but not in spontaneously hypertensive or normotensive rats.[8,9] Thus, relaxin apparently acts more rapidly in the renal circulation of normotensive rats than in the systemic vasculature (see below Influence of relaxin administration on the renal circulation in nonpregnant females and males).

Contribution of Relaxin to the Changes in Systemic Hemodynamics and Arterial Mechanical Properties during Pregnancy

A critical role for relaxin in the changes of systemic hemodynamics and arterial mechanical properties during midterm pregnancy in conscious rats was identified.[10] By administering relaxin neutralizing antibodies, the gestational increases in cardiac output and global arterial compliance, as well as the fall in systemic vascular resistance were abolished. Unexpectedly, no increase in passive compliance of isolated small renal or mesenteric arteries isolated from the midterm pregnant rats was observed. (But, this finding did corroborate earlier work.[11]) Nor was there any influence of the irrelevant control or relaxin neutralizing antibodies on arterial passive mechanics. Thus, the elevation in global arterial compliance at midgestation is a consequence of reduced vascular smooth muscle tone (i.e., vasodilation), increased number or branching of arteries, or increased passive compliance of arteries other than those investigated. Whether immuno-neutralization of circulating relaxin has complete or partial inhibitory effects during late pregnancy is presently under investigation. Possibly other hormones which are secreted by the placenta rather than the ovary contribute to systemic vasodilation (and osmoregulatory changes) during late gestation. Whether relaxin contributes to the changes in systemic hemodynamics and arterial mechanical properties during human pregnancy is currently unknown.

Because relaxin participates in both the relaxation and remodeling of the vascular wall (supra vide), there is overlap of hormonal and cellular signaling mechanisms for vasodilatory and arterial compliance changes. This sharing of hormonal and molecular mechanisms ensures a temporal coordination of the decline in both steady and pulsatile systemic arterial loads which, as previously mentioned, is critical to the maintenance of cardiovascular homeostasis during pregnancy.

During midterm pregnancy in the rat, relaxin contributes to arterial compliance changes by vasodilation, while in late pregnancy, relaxin may further increase arterial compliance by remodeling the vascular wall.[12,13]

Influence of Relaxin on the Renal Circulation

Renal Circulation during Pregnancy

In several serial studies throughout pregnancy in women, renal plasma flow (RPF) and glomerular filtration rate (GFR) were assessed by the renal clearances of para-aminohippurate and inulin, respectively (the "gold standards" for measurement of renal function, see reviews 14-16 and citations therein). On balance, these studies showed an increase in GFR and RPF of 40-65% and 50-85%, respectively, during the first half of gestation compared to prepregnant or postpartum values. Because the elevation in RPF exceeded that of GFR, the renal filtration fraction declined. During late gestation, a modest decline in RPF towards nonpregnant levels was reported, while GFR was maintained.The 24-h endogenous creatinine renal clearance is a reliable measurement of GFR in the setting of pregnancy (reviewed in ref. 14). Using this technique, Davison and Noble[17] showed that GFR rises 25% by the fourth gestational week (postLMP) compared to week 1 and reaches a 45% increase by week 9. Thus, the changes in renal hemodynamics are among the earliest and most marked maternal adaptations to pregnancy.

Animal models have been used to explore the underlying mechanisms. The Munich-Wistar rat has glomeruli belonging to the superficial cortical nephrons on the surface of the kidney that are accessible to renal micropuncture. Using this technical approach, Baylis demonstrated that the gestational rise in single nephron glomerular filtration rate was secondary to an increase in glomerular plasma flow with no change in glomerular hydrostatic pressure.[18] This constellation of events occurred as a consequence of parallel and similar decreases in the afferent and efferent renal arteriolar resistances. Although such an invasive approach cannot be used in humans, indirect evidence suggested comparable mechanisms for the gestational elevation in GFR.[19] Finally, because the gestational changes in renal hemodynamics and GFR in the chronically instrumented, conscious rat are comparable to human pregnancy, this animal model has been extensively investigated, in order to identify the underlying hormonal and molecular mechanisms.[20]

Relaxin was evaluated as a candidate hormone for the alterations in renal circulation, as well as osmoregulation during pregnancy. In pregnant rats and women circulating relaxin emanates from the corpus luteum of the ovary (reviewed in ref. 21). Human chorionic gonadotrophin (hCG) is an important stimulus for relaxin secretion during pregnancy in women.[21] In parallel with hCG, serum relaxin concentrations are highest during the first trimester (~1 ng/ml) and fall to lower levels in late gestation (~0.4 ng/ml;).[21] In fact, there were several compelling, albeit mainly circumstantial reasons to consider relaxin as the pivotal hormonal signal for triggering the circulatory and osmoregulatory changes of pregnancy as recently reviewed.[14,15]

Although renal vasodilation and hyperfiltration are detectable in gravid rats by gestational day 5, when serum relaxin is undetectable, there is a notable increase of renal function between gestational days 8 and 12 that coincides with a rise in ovarian and serum relaxin levels.[20,21] Thus, the increases in GFR and RPF that transpire in rat gestation before gestational 8 or during pseudo-pregnancy when circulating relaxin is undetectable may be mediated by other, as of yet, undetermined mechanisms. Alternatively, circulating relaxin concentrations below the level of assay detection may contribute.

Influence of Relaxin Administration on the Renal Circulation in the Nonpregnant Females and Males

Chronic, subcutaneous infusion of porcine relaxin or of rhRLX to chronically instrumented, conscious female rats for 2-5 days increased RPF and GFR (and reduced serum osmolality) to levels comparable with midterm pregnancy when renal function peaks in this species.[20,22] The renal vasodilatory response to relaxin was independent of the ovaries[22] and was also observed in male rats.[23] Chronic administration of relaxin also attenuated the renal vasoconstrictor response

to an acute angiotensin II infusion[22]—a phenomenon also observed during rat pregnancy.[24-26] Moreover, the myogenic reactivity of small renal arteries isolated from the relaxin-infused rats was inhibited[27] and similar to the inhibition previously shown in small renal arteries harvested from midterm pregnant rats.[11] In contrast to the lack of effect on systemic hemodynamics and arterial mechanical properties in normotensive rats as discussed above (Influence of relaxin administration on systemic hemodynamics and arterial mechanical properties in nonpregnant females and males), short-term administration of rhRLX to conscious rats for 1-4 hours produced significant renal vasodilation and hyperfiltration,[28] In normal human volunteers, short-term intravenous infusion of rhRLX for 6 hours increased RPF by 60%, but unexpectedly, not GFR.[29] The renal vasodilatory response was similar in men and women and transpired as soon as 30 minutes after starting the infusion of rhRLX. There were no significant decrements in blood pressure or serum osmolality. After 26 weeks of subcutaneous rhRLX infusion in patients with mild scleroderma, the predicted creatinine clearance rose by 15-20% and serum osmolality and blood pressure fell slightly, but significantly throughout the study in a dose dependent fashion.[30]

Contribution of Relaxin to the Changes in the Renal Circulation during Pregnancy

By administering relaxin neutralizing antibodies or removing circulating relaxin by ovariectomy while maintaining the pregnancy with exogenous estrogen and progesterone, renal hyperfiltration, vasodilation and reduced myogenic reactivity of small renal arteries were abolished in midterm pregnant rats.[5] The osmoregulatory adaptations to pregnancy were also inhibited.[5] Thus, relaxin is essential for the renal circulatory and osmoregulatory alterations during midterm pregnancy in rats.

In women who lacked ovarian function and became pregnant through egg donation, IVF and embryo transfer, the gestational rise in GFR and fall in serum osmolality were significantly subdued.[31] Because these women lacked functioning ovaries, serum relaxin was undetectable. Thus, comparable to gravid rats, endogenous circulating relaxin most likely plays a role in the initiation of the renal and osmoregulatory responses to pregnancy in women. However, unlike the gravid rat, partial responses may persist despite the absence of detectable circulating relaxin.

Cellular Mechanisms of Vasodilation

An emerging view is that the vasodilatory mechanisms of relaxin vary according to the time of exposure to the hormone, i.e., there are slow and fast vasodilatory responses.

Slow Responses (Fig. 1)

As reviewed recently, nitric oxide (NO) mediates renal vasodilation and hyperfiltration in the gravid rat model.[14-16] In contrast, the vasodilatory prostaglandins play little or no role.[14-16] In addition, NO mediates the loss of myogenic reactivity in small renal arteries obtained from midterm pregnant rats.[11] This NO dependency was also found for the renal vasodilation and hyperfiltration and loss of myogenic reactivity in small renal arteries caused by chronic relaxin administration to nonpregnant rats.[22,27] The mechanism for NO-dependent changes in the renal circulation of midterm pregnant rats or of relaxin-infused nonpregnant rats is not due to increased expression of endothelial nitric oxide synthase.[32,33]

During midterm pregnancy or during chronic subcutaneous infusion of rhRLX to nonpregnant rats, *endothelin* (ET) mediates the renal vasodilatory changes through stimulation of the endothelial ET_B receptor subtype which is linked to NO production (reviewed in refs. 14-16). Whether the expression of endothelial ET_B receptors increases, thereby constituting a primary event that mediates renal vasodilation, hyperfiltration and reduced myogenic artery of small renal arteries during pregnancy or after chronic administration of rhRLX to nonpregnant rats is presently controversial (refs. 34,35 and see below Influence of relaxin on cultured vascular cells; *The endothelial ET_B receptor*).

Typically, the traditional endothelin converting enzymes that are antagonized by phosphoramidon mediate the processing of big ET, an inactive precursor, to ET_{1-21}. However, phosphoramidon

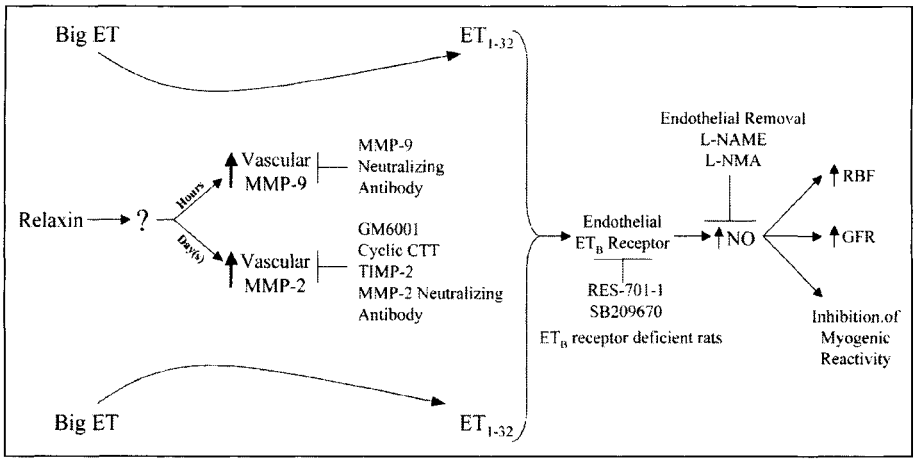

Figure 1. Working model for the slow vasodilatory actions of relaxin. ?, indicates the likelihood of another intermediary molecule. ⊣, indicates inhibitors of relaxin vasodilation. ET, endothelin; MMP, matrix metalloproteinase; RBF, renal blood flow; GFR, glomerular filtration rate; GM6001, a general MMP inhibitor; cyclic CTT, a specific peptide inhibitor of MMP-2; TIMP-2, tissue inhibitor of metalloproteinase; RES-701-1, a specific ET_B receptor antagonist; SB209670, a mixed ET_A and ET_B receptor antagonist; L-NAME, nitro-L-arginine methyl ester; L-NMA, N^G-monomethyl-L-arginine. Note that phosphoramidon (an inhibitor of the classical endothelin converting enzyme), STT (control peptide for cyclic CTT); heat inactivated TIMP-2, BQ-123 (a specific ET_A receptor antagonist) and IgGs (control antibodies for MMP neutralizing antibodies) did not affect the slow vasodilatory actions of relaxin. See text for details and references.

did not affect renal vasodilation, hyperfiltration or reduced myogenic reactivity of small renal arteries harvested from nonpregnant rats chronically administered rhRLX.[36] Therefore, based on the confluence of our own and others experimental findings, we hypothesized that relaxin may increase vascular *matrix metalloproteinase-2* (MMP-2) activity in renal arteries during pregnancy that, in turn, cleaves big ET to ET_{1-32} at a glycine-leucine bond, thereby accentuating the endothelial ET_B receptor-NO vasodilatory pathway.[36]

After acute infusion of a specific inhibitor of gelatinase (MMP-2 or -9) or of a general inhibitor of MMP to conscious rats during chronic administration of rhRLX, renal vasodilation and hyperfiltration were abrogated. Furthermore, the absent myogenic reactivity of small renal arteries obtained from midterm pregnant rats or from nonpregnant rats chronically administered rhRLX was restored by incubating the arteries with the specific MMP-2 or -9 inhibitor, the general MMP inhibitor, TIMP-2 (tissue inhibitor of metalloproteinase), or a specific MMP-2 neutralizing antibody, but not by phosphoramidon. In addition, MMP-2 activity was augmented in small renal (and mesenteric) arteries isolated from midterm pregnant rats or nonpregnant rats chronically administered the hormone. An elevation in MMP-2 activity in response to chronic subcutaneous rhRLX infusion was also seen in small renal arteries from rats that were genetically deficient in the ET_B receptor. However, myogenic reactivity remained robust and was not the least bit attenuated in these arteries. The latter finding corroborated the essential role of the endothelial ET_B receptor in mediating the inhibition of myogenic reactivity by rhRLX as previously determined using pharmacological inhibitors of the receptor.[14-16] Similar results were observed in midterm pregnant rats deficient in the ET_B receptor.[37] Of greater significance, however, is the dissociation of increased vascular MMP-2 activity from the inhibition of myogenic reactivity strongly suggests that MMP-2 is in series with and upstream of, the endothelial ET_B receptor-NO vasodilatory pathway).

The mechanism(s) underlying the increase in vascular MMP-2 activity by chronic rhRLX administration or pregnancy is incompletely understood. However, both pro and active MMP-2

activities are elevated by a similar degree, MMP-2 protein and mRNA are also increased and there are no significant alterations in TIMP-1 and 2.[38] MMP-2 protein is observed in both endothelium and smooth muscle, but further investigation is required to determine in which of these vascular compartment(s) it increases in response to pregnancy or chronic rhRLX administration. Although it is likely that the relaxin LGR7 receptor mediates the arterial responses to pregnancy and chronic relaxin administration in nonpregnant rats, this supposition needs testing. Furthermore, whether the relaxin receptor which mediates the slow vasodilatory responses is located in the endothelium or smooth muscle and whether there are intermediary molecules linking relaxin receptor activation to increased MMP-2 expression are presently unknown.

Recently, we reported that *matrix metalloproteinase-9* (MMP-9) rather than MMP-2 activity is elevated in small renal and mesenteric arteries isolated from rats after short-term subcutaneous infusion of rhRLX for 4-6 hours.[39] Small renal arteries demonstrated loss of myogenic reactivity and robust myogenic reactivity was restored by incubation with a specific MMP-9 antibody, rather than a specific MMP-2 antibody as observed following day(s) of rhRlx administration (supra vide). Like MMP-2, MMP-9 can process big ET at a glyine-leucine bond. The inhibition of myogenic reactivity after 4-6 hours of rhRLX administration was also mediated by the endothelial ET_B receptor-nitric oxide vasodilatory pathway. Preliminary studies suggest that these slow vasodilatory responses after hours to day(s) of hormone exposure require cross-talk between the endothelium and vascular smooth muscle involving vascular endothelial growth factor (VEGF) in one direction (vascular smooth muscle to endothelium), in addition to nitric oxide in the other direction (endothelium to vascular smooth muscle).[40]

Fast Responses

Arteries isolated from human gluteal biopsies and mounted in a wire myograph, relaxed to rhRLX after preconstriction with norepinephrine in an endothelium-dependent fashion. However, human pulmonary resistance arteries did not vasodilate in response to rhRlX.[41] Human subcutaneous arteries studied in a pressure arteriograph and preconstricted with phenylephrine demonstrated a rapid and marked vasorelaxation to rhRLX that was mediated by phosphatidylinositol 3-kinase and NO.[42] Similar findings were observed for rat small renal, but not mesenteric or coronary (septal) arteries.[42] Thus, the ability of relaxin to produce rapid relaxation in isolated human and rat blood vessels is apparently dependent on their anatomical source.

Influence of Relaxin on Cultured Vascular Cells

Inducible Nitric Oxide Synthase

In cultured vascular smooth muscle cells from bovine aorta, Bani et al provided evidence that purified porcine RLX induced iNOS expression.[43] The porcine relaxin was stated to be endotoxin-free as determined by the Limulus amebocyte lysate assay. They reported that porcine RLX stimulated calcium and calmodulin-independent NOS activity, upregulated iNOS protein by immunohistochemistry, elevated nitrite in the conditioned media, increased cGMP in the cultured cells and blocked the rise in the calcium transient by thrombin. The same investigators utilized similar methodological approaches to show that porcine RLX could upregulate iNOS in cultured rat coronary artery endothelial cells[44] and in cultured human umbilical artery endothelial cells.[45] Whether RLX induces iNOS in vivo was not investigated, although the authors presented data supporting iNOS induction by RLX in hearts challenged with anaphylaxis (normal hearts were apparently not investigated, see below Relaxin and cardiac disease; *Cardiac anaphylaxsis*).

It should be pointed out that not all evidence supports the concept that relaxin induces iNOS expression insofar as (i) infusion of porcine RLX to conscious rats failed to increase the urinary excretion of cGMP and of nitrate plus nitrite,[22] (ii) calcium-independent NOS activity was not increased in isolated mesenteric arcades or thoracic aortae from rats of gestational days 15-17 when circulating levels of relaxin are high[46] and (iii) iNOS expression was not induced in renal tissues by pregnancy (or rhRLX) as evaluated by Western analysis at least in one study,[32] although it may have been in another.[33]

The Endothelial Endothelin B Receptor

Dschietzig and coworkers reported that human RLX augmented the expression of the ET_B receptor subtype in cultured human umbilical artery endothelial cells.[35] The hormone elevated ET_B receptor mRNA, protein and binding sites for radiolabeled ET-1. RLX activated extracellular signal-regulated kinases-1/2 (ERK-1/2), but not p38 kinase and enhanced the DNA binding activity of the transcription factor, nuclear factor NF-κB; pharmacological blockade of these signal transduction pathways prevented the elevation in endothelial ET_B receptor mRNA expression. Many of these findings were duplicated in cultured bovine aortic endothelial cells. The expression of the ET_B receptor by human vascular smooth muscle cells was unaffected by RLX, nor was the ET_A receptor subtype altered in any of the cultured cells by the hormone. Relaxin also increased NF-κB reporter activity in bovine aortic endothelial cells which was not affected by dominant negative Ras (a GTP binding protein coupled to growth factor receptors), but was inhibited by dominant negative forms of mitogen activate protein kinase kinase-1 (MEK-1), Raf-1 (a MEK kinase) and ERK-1/2, thereby providing further clues to the cell signaling mechanisms. The functional importance of these findings was shown in arteries from rats, insofar as incubation with RLX in vitro resulted in a decrease in the sensitivity and maximal response to ET-1-induced contraction. Furthermore, the vasorelaxation to ET-3 was enhanced. All of these functional effects were blocked by the ET_B receptor antagonist, A-192621 or by the MEK-1 inhibitor, PD-98059. On balance, these results corroborate those obtained from the renal circulation where pregnancy or relaxin-treatment of nonpregnant rats elicited renal vasodilation, hyperfiltration and impaired myogenic reactivity of small renal arteries via the endothelial ET_B receptor/NO vasodilatory pathway.[5,11,23-25,27,36,47] The study of Dschietzig and colleagues further suggests that one potential mechanism for this vasodilatory role of RLX is through upregulation of the endothelial ET_B receptor.

The concept that relaxin upregulates the endothelial ET_B receptor is both logical and attractive and Kerchner et al also tested the hypothesis. Unfortunately, they were unable to find any supportive evidence.[34] Briefly, ET_B expression was investigated in small renal arteries that were harvested from virgin and midterm pregnant rats, as well as from nonpregnant rats that were infused subcutaneously with recombinant human relaxin (rhRLX) or vehicle for 5 days or for 4-6 hours. Small renal arteries harvested from additional virgin rats were incubated with rhRLX or vehicle for 3 hours at 37°C in vitro. ET_B expression was also assessed in cultured human endothelial cells: aortic (HAEC), coronary (HCAEC), umbilical vein (HUVEC) and dermal microvascular endothelial cells (HMVEC). Cells were incubated for 4, 8, or 24 hours with rhRLX (5, 1, or 0.1ng/ml) or with vehicle. ET_B protein in arteries and cells was evaluated by Western analysis. No alteration of ET_B expression was observed in small renal arteries from any of the experimental protocols. Nor was there an augmentation of the vasorelaxation response to ET-3 in small renal arteries incubated in vitro with rhRLX. rhRLX only sporadically altered ET_B expression in HCAEC and HUVEC at certain time points or doses and no regulation was observed in HAEC or HMVEC. These results suggest that up-regulation of ET_B receptor protein has little or no role in relaxin stimulation of the endothelial ET_B/NO vasodilatory pathway. Rather, the pivotal step regulated by relaxin is vascular gelatinase activity (see above Cellular mechanisms of vasodilation; *Slow responses*).

Influence of Relaxin on the Circulations of Other Organs

Coronary Blood Flow

Bani-Sacchi and colleagues demonstrated that relaxin was a potent coronary vasodilator.[48] Hearts were harvested from male guinea pigs and rats, mounted in a Langendorff apparatus and perfused retrograde through the aorta at constant pressure. The coronary effluents were collected for determination of coronary flow and nitrite (a metabolite of NO). Whether administered by bolus injection into the aortic cannula (1, 5 and 10 nM) or by addition to the perfusion media for constant infusion (5 nM), there was a fast and parallel increase in coronary blood flow and nitrite concentration. Both were prevented by pretreatment with 0.1 mM L-NMMA, an inhibitor of NO synthase. In these protocols, relaxin was found to be more potent than acetylcholine (plus physostigmine to inhibit acetylcholinesterase) and sodium nitroprusside, endothelium-dependent and –independent vasodilators, respectively.

Uterus

The first evidence suggesting that relaxin affects blood vessels was based on histological studies conducted in the uterus. In 1966, Dallenbach-Hellweg et al reported an enlargement of arterioles and capillaries in the superficial part of the endometrium of castrated monkeys treated with RLX.[1] In a later study also in monkeys, they described not only an enlargement of the vessels but also endothelial cell proliferation[2] Vasilenko and colleagues published similar findings in ovariectomized rats administered porcine RLX.[49] Finally, immunohistochemical studies demonstrated binding sites for RLX on cells associated with blood vessels in the uterus, cervix and vagina of pigs and humans.[50,51] These studies are consistent with a vasodilatory role for RLX in the uterine circulation.

The results of functional studies, however, are less clear. rhRLX treatment has been shown to increase uterine blood flow in conscious ovariectomized female rats.[52] In contrast, porcine RLX had no effect on endometrial and myometrial blood flow in mature anesthetized sheep.[53] It is likely that species differences explain this discrepancy. Sheep do not produce RLX because the mRNA contains numerous stop codons in the C-peptide region which prevent the translation of a functional RLX molecule.[54] To our knowledge, there are no studies directly examining the effects of RLX on uterine blood flow in humans. However, in phase II/III trial of rhRLX in the treatment of scleroderma, the most frequently reported adverse event by women receiving the hormone was heavy or irregular menstrual bleeding suggesting increased endometrial vascularization. In addition, rhRLX increases expression of VEGF in human endometrial cells in culture.[55] These findings support a role for RLX in the regulation of human endometrial blood flow. In contrast, a study in pregnant women showed a positive correlation between plasma RLX levels and the resistance or pulsatility index measured by ultrasound early in gestation. This finding suggests that RLX may increase uterine artery resistance during pregnancy.[56] Finally, in an in vitro study of human intramyometrial arteries, rhRLX had no effect on either resting tension or tension induced by U46619 (a thromboxane analog), endothelin or PGF_{2a}[57]. A role for RLX in the modulation of uterine blood flow requires further investigation.

Placenta

RLX binding sites were found on blood vessels in the chorionic plate and within the placental villi.[50] Despite the fact that binding sites for RLX were identified, rhRLX did not affect resting tension or tension induced by U46619, endothelin-1 or $PGF_{2\alpha}$ in human placental stem villous arteries studied in vitro.[57] Another study involving human umbilical arteries failed to demonstrate an effect of RLX or RLX and progesterone on serotonin and KCl stimulated contractions in vitro.[58]

Mammary Gland

Evidence that RLX may act as a vasodilator in the mammary circulation includes morphometric analyses of microvessel lumina from ovariectomized mice 18-20 hours after a single injection of porcine RLX. In this study, the mean diameters of arterioles, capillaries and postcapillary venules in the mammary glands of the RLX-treated mice were significantly greater when compared to ovariectomized control mice.[59] It is possible that this effect of RLX is not limited to the mouse since RLX binding sites were identified in blood vessels of the human mammary gland and nipple.[50,51] The pigeon crop sac is a structure analogous to the mammary gland and there are several advantages to studying this organ. One is that hormones can be administered by intradermal injection which permits study of local effects. Another advantage is that the anatomy of the crop sac allows for treatment of one hemi-crop, while the contralateral crop serves as control. In this tissue, there was striking dilation of the blood vessels in the lamina propria of mucosa 6 hours after injection of porcine RLX in one hemi-crop compared to the vehicle-treated contralateral crop.[60]

Liver, Mesentary and Mesocaecum

The influence of RLX on the hepatic vasculature was investigated in male rats. In this study, porcine RLX dilated the liver sinusoids. This effect was reduced by NO synthesis inhibition.[61]

In the mesenteric circulation, RLX modulated the vascular responses to vasoconstrictor agents. In the perfused mesentery of the spontaneously hypertensive rat in situ, the vasoconstrictor responses

to arginine vasopressin and norepinephrine were attenuated after a 42 hour infusion of purified rat RLX.[62] In the same investigation, the sensitivity to norepinephrine was also reduced in the isolated portal vein from RLX-treated rats while the sensitivity to angiotensin was unchanged. After a six hour incubation of isolated mesenteric and renal arteries, as well as aorta from rats with rhRLX, the maximal contractile response and sensitivity to ET-1 decreased while the relaxation response to ET-3 was augmented.[35] (ET-1 contracts arteries by interacting with ET_A and/ET_B receptor subtypes on vascular smooth muscle. ET-3 relaxes arteries by interacting with the ET_B receptor subtype on endothelium.) These functional responses suggested that RLX upregulates the endothelial ET_B receptor, thereby offsetting the contractile response to ET-1 and potentiating the relaxation response to ET-3 (also see above Influence of relaxin on cultured vascular cells; *The endothelial ET_B receptor*). Mesenteric arteries isolated from rats after chronic administration of rhRLX were also less responsive to changes in intraluminal pressure (reduced myogenic reactivity) compared to vehicle treated rats when studied in a pressure arteriograph system.[27]

In the mesocaecum, administration of porcine RLX to male Wistar rats elicited a rapid dose-dependent dilation of the veins; however, arteriolar and capillary flows were unchanged.[63] In the same study, RLX administration also opposed the vasospasm induced by norepinephrine or promethazine (an anticholinergic agent) in the arteries of the mesocaecum.

Relaxin and Angiogenesis

Vasodilatory hormones frequently have an angiogenic role (or vice versa). One of the many interesting observations to arise from the phase II/III trial of rhRLX in the treatment of scleroderma was the high incidence of menometrorrhagia (heavy, irregular or prolonged menstrual bleeding) in those women administered hormone.[55] In cultured human endometrial cells, rhRLX stimulated the production of VEGF in a cAMP-dependent fashion.[55] rhRLX stimulated new blood vessel formation in the endometrium of a nonhuman primate model of early pregnancy.[64] Others have also reported the angiogenic properties of relaxin in various settings.[65-67] Taken together, these results suggest that relaxin contributes to neovascularization.

Unemori and colleagues reported enhanced vessel in-growth of Matrigel plugs containing rhRLX that were implanted subcutaneously in mice.[68] Using the Hunt-Schilling wound chamber assay, they documented increased VEGF 164 and basic fibroblast growth factor mRNA expression by cells (presumably macrophages) in the chamber aspirate of those rats administered rhRLX systemically. This increase corresponded with enhanced expression of factor VIII related antigen in the granulation tissue of the chamber, indicative of enhanced blood vessel growth. Interestingly, VEGF mRNA expression was not elevated by rhRLX in resident macrophages of the lung or spleen remote from the site of injury. Nor did rhRLX have a direct angiogenic effect on cultured endothelial cells confirming an earlier report.[69] Finally, rhRLX was also shown to increase VEGF 165 and 120, as well as bFGF mRNA expression by THP-1 cells, a human monocyte/macrophage cell line. Taken together, rhRLX appears to be indirectly angiogenic by enhancing VEGF and bFGF expression in wound macrophages, thereby stimulating angiogenesis. In a subsequent investigation by the same investigators, systemic administration of rhRLX was demonstrated to significantly accelerate wound closure in full thickness skin excisions in db/db mice.[70] This accelerated healing was associated with increased staining for factor VIII related antigen in the granulation tissue which was thicker and more cellular than in vehicle-treated mice.

Further evidence for the indirect angiogenic attributes of rhRLX was shown in a model of chronic myocardial infarction in rats.[71] Systemic infusion of rhRLX potentiated the increase in bFGF mRNA and protein at 7 and 21 days in the peri-infarct region where the growth factor was expressed by myocytes and fibroblasts. rhRLX infusions in sham operated rats showed no change in bFGF or VEGF expression in corresponding regions of the left ventricle or in the right ventricle. The increase in the number of thin-walled, collateral vessels lacking smooth muscle was correspondingly potentiated in the peri-infarct region by systemic rhRLX. In the same publication, rhRLX was reported to increase the mRNA and protein for VEGF and bFGF in human neonatal heart cells in culture (fibroblasts and myocytes).

Evidence for a Vascular-Derived Relaxin Ligand-Receptor System

We explored whether there is local expression and function of relaxin and its receptor in arteries of nonpregnant females and males.[72] Relaxin-1 and its major receptor, LGR7, mRNA were expressed in thoracic aortae, small renal and mesenteric arteries from mice and rats of both sexes, as well as in small renal arteries from female tammar wallabies (an Australian marsupial). Using antibodies available for rat and mouse LGR7 receptor and rat relaxin, we also identified protein expression in arteries. These results extended the findings of Dscheitzig et al who showed H1 and H2 relaxin mRNA expression in human saphenous vein and mammary artery,[73] as well as those of Sherwood and colleagues who demonstrated relaxin binding sites on blood vessels in reproductive organs.[50,51] Small renal arteries harvested from relaxin-1 gene deficient mice demonstrated enhanced myogenic reactivity and decreased passive compliance relative to wild-type and heterozygous mice.[72] Taken together, these findings suggest an arterial-derived, relaxin ligand-receptor system that acts locally to regulate arterial function.

Part 2. Relaxin and Vascular Dysfunction: Implications for Relaxin as a Therapeutic Agent (Table 2)

Relaxin and Renal Disease

Given the renal vasodilatory (see above Influence of relaxin on the renal circulation) and matrix-degrading[74-76] properties of relaxin, there are important therapeutic implications of this hormone in disorders associated with renal vasoconstriction and fibrosis. While scarring and fibrosis are late findings of renal disease, renal alterations may occur prior to clinically recognized renal insufficiency. At this time period, the hemodynamic effects of relaxin may also have therapeutic benefit. As described above, relaxin administration, similar to pregnancy, results in renal vasodilation and hyperfiltration in rats via an endothelial endothelin B and nitric oxide pathway in rats. Renal micropuncture in pregnant Munich-Wistar rats by Baylis and colleagues indicates that the vasodilation of the efferent and afferent arterioles occurs without a net increase in glomerular capillary pressure.[18] Thus, relaxin may have therapeutic implications in renal disorders characterized by increased intraglomerular pressure secondary to efferent arteriolar constriction. Smith and colleagues administered intravenous rhRLX to healthy male and female volunteers over 6 hours and noted a 47% increase in renal plasma flow, but no significant change in glomerular filtration rate.[29] There were no adverse effects suggesting that relaxin may be used in humans and could have potential therapeutic benefit in increasing renal blood flow in certain disease states. Longer infusions may also increase GFR.[22,30]

Despite varying etiologies of progressive renal diseases, scarring is the final common pathway leading to renal insufficiency, end stage renal disease, dialysis and need for transplantation. Fibrosis can affect both the renal parenchyma and vascular components of the kidney leading to tubulointerstitial scarring and glomerulosclerosis, respectively. The finding that relaxin-deficient mice (RLX -/-) develop an age-related progressive fibrosis in the kidney and other tissues[77] underscores the role of relaxin in the regulation of matrix turnover and fibrosis. Furthermore, exogenous administration of relaxin in several rodent models of renal disease results in improvement; these include bromoethylamine-induced renal interstitial fibrosis,[78] ablative and infarction models of 5/6 nephrectomy,[79] nephrotoxicity related to cyclosporine,[80] anti-glomerular basement membrane nephritis (Goodpasture's syndrome),[81] and RLX -/- renal fibrosis.[82] Danielson and colleagues demonstrated that rhRLX administration to aged Munich Wistar rats improved both renal function and histology.[83] GFR and ERPF were increased and effective renal vascular resistance decreased with long-term RLX-infusion over days. Double-blinded histologic examination of the kidneys revealed a decrease in glomerular and tubular collagen deposition in the RLX-treated compared to vehicle-treated controls. With short-term (24 hours) RLX-administration, the improved renal function was mediated by vascular gelatinase activity. This was demonstrated by infusion of a specific gelatinase inhibitor, cyclic CTT and a general MMP inhibitor, GM6001, as well as upregulation of vascular MMP-2 activity on gelatin zymography (see above Cellular mechanisms of vasodilation).

Table 2. Relaxin and vascular dysfunction: potential therapeutic implications

Target Organ System	Specific Disease Process	Therapeutic Benefi
Kidneys	Renal vascular disease—vasodilation	• Vasodilation of pre- and post-glomerular arterioles without increase in intraglomerular pressure in rats (18) • Increased renal blood flow in healthy male and female volunteers with IV RLX (29) • Increased predicted creatinine clearance in patients with mild scleroderma during chronic subcutaneous RLX administration (30)
	Renal fibrosis	• Age-related renal fibrosis in RLX-deficient mice(77) • Bromoethylamine-induced renal fibrosis (78), models of 5/6 nephrectomy (79), cyclosporine nephrotoxicity (80), anti-glomerular basement membrane nephritis (81) • Renal histology and function in aged rats (83)
Heart	Myocardial ischemia-reperfusion (I/R) injury	• I/R with coronary artery ligation in guinea pig, rat and swine models (85, 86,93,94) • Isoproteronol-induced myocardial ischemia in rats (92)
	Cardiac fibrosis	• Age-related cardiac fibrosis in male RLX -/- mice (95) • β2 adrenergic receptor overexpressing transgenic mice (96) • Spontaneously hypertensive rats (99,100)
	Heart failure	• Afterload reduction (6,8-10)
	Post-infarction heart	• Improved integration of transplanted cells in permanently damaged heart – in vitro coculture model (94,106)
	Cardiac anaphylaxis	• Protection against cardiac anaphylaxis in ex vivo guinea pig heart model (107)
Brain	Cerebral ischemia (stroke)	• Rodent model of stroke (middle cerebral artery occlusion) (108)
Gastrointestinal tract	Intestinal ischemia-reperfusion	• Rat model with splanchnic artery occlusion (111)
	Liver transplantation—preservation and reperfusion	• Isolated perfused rat liver model (112)
Pulmonary	Pulmonary hypertension	• Hypoxia model of pulmonary hypertension in rats (113)
Blood vessels	Vascular inflammation	• In vitro co-culture model of coronary artery endothelial cells and neutrophils (88)
Pregnancy	Preeclampsia	• Potential benefit based on angiogenic effects, renal and systemic vasodilation in rats and humans (above) and uterine artery vasodilation in rats (52)

Interestingly, after 20 days, the improved renal function was insensitive to gelatinase inhibition, suggesting a permanent alteration in the vascular structure.

The anti-fibrotic mechanisms of relaxin and potential therapeutic benefits of relaxin are discussed further in the chapter by Dr. Samuel and coworkers.

Relaxin and Cardiac Disease

In addition to systemic and renal vasodilatory effects, relaxin has direct cardiac effects that may be important in both health and disease. Relaxin is produced by the heart[73,84] and acts on specific cardiac receptors. There are also direct positive chronotropic and inotropic effects on the heart (at least in rodents), as well as platelet inhibitory effects of relaxin that may play a role in cardiovascular health and disease.

Myocardial Ischemia-Reperfusion Injury

Masini and colleagues demonstrated that administration of exogenous relaxin improves myocardial injury secondary to ischemia-reperfusion in a rodent model.[85] Using isolated hearts from male guinea pigs, myocardial ischemia was produced by ligating the left anterior descending coronary artery. When porcine relaxin (30 ng/ml) was added to the perfusate at the start of coronary occlusion, coronary flow ligation was preserved despite ligation most likely secondary to dilation of the extensive collateral circulation in the guinea pig heart. In addition, RLX augmented coronary flow during reperfusion. Furthermore, nitrite concentration was increased in the coronary effluent, histamine release from mast cells was reduced, markers of oxygen free-radical mediated cardiomyocyte injury including malondialdehyde and calcium content were also reduced in the relaxin-treated hearts.

Studies of the beneficial effects of relaxin in cardiac ischemia-reperfusion injury were extended to an in vivo rat model by Bani and colleagues.[86] Thoracotomy was performed on anesthetized rats and the left anterior descending coronary artery was ligated for 30 minutes followed by 60 minutes of reperfusion. Rats administered porcine RLX (100 ng intravenously) 30 minutes prior to occlusion had significantly reduced area of myocardial damage, fewer ventricular arrhythmias and reduced mortality. In addition, neutrophil accumulation, lipid peroxidation, calcium content, endothelium and myocyte injury, mast cell degranulation and histamine release were all reduced with relaxin pretreatment. This group of investigators has also posited that relaxin may have direct effects on neutrophils to prevent activation and adhesion to endothelial cells,[87,88] on mast cells to inhibit degranulation and histamine release,[89,90] on myocytes to reduce calcium metabolism and on platelets to reduce aggregation[91] possibly via a nitric oxide-mediated mechanism.

Using a rat model of isoproterenol-induced myocardial ischemia, Zhang and colleagues[92] demonstrated increased rat relaxin-3 protein and mRNA in the myocardium and increased plasma relaxin-3 (the latter measured by RIA from Phoenix Pharmaceutical Inc.). Furthermore, administration of exogenous relaxin (0.2 and 2.0 μg/kg/day) improved cardiac function, reduced levels of malondialdehyde, lactate dehydrogenase and creatine phosphokinase in the plasma of relaxin-treated rats compared to controls. With the higher dose of relaxin, fibroblastic hyperplasia in the myocardium and circulating endothelin were reduced. These findings suggest a protective role for endogenous relaxin and a therapeutic role for exogenous relaxin in this model of myocardial injury.

More recently, the therapeutic potential of recombinant human relaxin in myocardial ischemia-reperfusion injury has been extended to a swine model[93,94] which has been used extensively to test cardiac drugs and therapies due to intrinsic similarities to human ischemic heart disease. In this approach, the investigators sought to mimic the clinical situation of acute myocardial infarction followed by admission to an intensive care setting and intervention with percutaneous coronary artery angioplasty. The current standard of care for acute myocardial infarction includes catheter-based reperfusion with either balloon angioplasty with or without coronary artery stenting. Preventing reperfusion injury with relaxin is the basic hypothesis of these most recent studies. Using the swine model, thoracotomy and catheterization was performed under anesthesia followed by occlusion of the left anterior descending artery for 30 minutes.[93] Relaxin was administered for

20 minutes starting at reperfusion. Three doses of RLX, 1.25, 2.5 and 5 μg/kg bodyweight, were utilized. At the two higher doses, there was reduction of the main serum markers of myocardial damage (myoglobin, creatine kinase—MB fraction, troponin T), reduced tissue parameters of oxygen free-radical induced cardiomyocyte injury (malodialdehyde, tissue calcium), reduced cardiomyocyte apoptosis (caspase 3) and reduced inflammatory leukocyte recruitment (myeloperoxidase) compared to vehicle-treated controls. Furthermore, there was a reduction in the tissue volume that did not uptake the tracer ^{201}Thallium and less ultrastructural evidence of cardiac damage. Cardiac contractile performance was also improved at the 5 μg/kg dose of relaxin.

Cardiac Fibrosis

A variety of causes result in cardiac fibrosis including but not limited to acute inflammation, myocardial infarction, aging, increased hemodynamic load, neurohumoral activation and metabolic disorders. Hypertensive disorders and ischemic heart disease resulting in cardiac fibrosis and/or hypertrophy are of particular clinical importance. Evidence suggests that endogenous relaxin may modulate the extracellular matrix turnover and scarring and exogenously administered relaxin may have therapeutic benefit. This topic is reviewed extensively by Samuels and colleagues elsewhere in this book, but will be summarized briefly here.

Male relaxin-deficient (RLX -/-) mice demonstrate an age-related progression of cardiac fibrosis.[95] Increased left ventricular collagen content and concentration as well as increased atrial hypertrophy, left ventricular procollagen I mRNA expression, chamber stiffness and diastolic dysfunction were noted in relaxin-deficient mice compared to wild-type controls from nine months of age onwards.[95] Interestingly, these features were not observed in relaxin-deficient female mice. Local expression of relaxin and relaxin receptors supports the idea of the cardiac tissue as a potential source and/or target for relaxin. Using RT-PCR, relaxin and relaxin-3 gene transcripts have been identified in the atria and ventricles of rodent hearts[95-97] and H1 and H2 RLX are constitutively expressed in human cardiovascular tissues and upregulated in disease states such as heart failure.[73] Binding sites have also been identified in the atrium of male and female rats.[98]

Exogenous relaxin has cardiac anti-fibrotic properties in various rodent models. Treatment of established cardiac fibrosis in 12 month old RLX -/- mice with two weeks of rhRLX reduced collagen deposition.[96] Infusion of rhRLX for 14 days reversed cardiac fibrosis in transgenic animals overexpressing β2 adrenergic receptors which eventually develop heart failure.[96] In the spontaneously hypertensive rat model, rhRLX administration to 9-10 month old rats significantly decreased collagen content in the myocardium of the left ventricle, but not the unaffected chambers of the heart.[99] Another group of investigators demonstrated left heart hypertrophy and selective elevation of left atrial and ventricular relaxin gene expression and relaxin peptide in 12-month old spontaneously hypertensive rats.[100] Work in the arena of cardiac fibrosis suggests that endogenous relaxin may have a protective role in cardiac injury and fibrosis and exogenous relaxin may have a therapeutic role in preventing or reducing fibrosis in the heart. Further investigation is needed before extrapolating these findings to human disease.

Heart Failure

Dschietzig and colleagues demonstrated an association between congestive heart failure (CHF) and circulating relaxin levels in humans.[73] Circulating RLX levels correlated with disease severity compared to controls. In 11 of 14 subjects with severe CHF, the RLX concentration in the coronary sinus was greater than the left ventricle suggesting a cardiac contribution to the elevated circulating RLX levels. Furthermore, after 12 to 48 hours of sodium nitroprusside infusion, plasma RLX levels in severe CHF patients fell to those seen in moderate CHF. There was no change in RLX with nitroprusside infusion in the moderate CHF group. RLX levels also correlated with left ventricular end diastolic pressure ($r = 0.69$) and cardiac index ($r = -0.62$). In tissues from the failing heart, these authors noted increased H1 and H2 gene expression in the right atrium and left ventricle, increased 18-kDa prorelaxin but not the 6-kDa mature form in the right atrium. Interestingly, levels of the prohormone convertase-1 mRNA were decreased in the right atrium but not the left ventricle possibly accounting for the increase in prorelaxin in the atrium but not

the ventricle. It should be noted, however, that not all investigators observed an association between circulating levels of relaxin and heart failure[101] (and see Controversies, Unresolved Issues and Future Directions). Based on the unique constellation of effects in the systemic and renal circulations, relaxin may be a particularly effective therapeutic agent for afterload reduction in congestive heart failure.[6,16]

Post-Infarction Heart

A newer research area in the therapeutic potential of relaxin has been in the post-infarction heart and permanently damaged tissue, including scarring and aneurysms. There has been interest in precursor cell grafting to repair the post-infarct myocardium.[102,103] Currently, skeletal muscle myoblasts have garnered particular attention because of high tolerance to ischemia, high proliferative potential and autologous source.[103-105] On the basis of relaxin's anti-fibrotic effects, angiogenic potential and influence on cardiomyocytes in fetal and neonatal periods, Bani and colleagues have performed preliminary studies using relaxin to improve integration of the grafted cells into the host cardiac tissue.[94,106] Using in vitro coculture of mouse skeletal myoblasts and adult rat cardiomyocytes, relaxin increased gap junction formation with increased connexin 43 expression on myoblasts and potentiated gap junction-mediated intracellular exchanges including increased conductance and intercellular transmission of calcium signals. These preliminary investigations suggest a novel role for relaxin in promoting effective transplantation and differentiation of cells into the damaged heart.

Cardiac Anaphylaxis

Masini and coworkers have also shown a beneficial effect of RLX in protecting against cardiac anaphylaxis in an ex vivo guinea pig heart model.[107] Two intraperitoneal injections of ovalbumin were administered on consecutive days to male guinea pigs for the purpose of sensitization. Fifteen to 30 days later, the heart was harvested and studied ex vivo using a Langendorff apparatus. Cardiac anaphylaxis was induced by administration of ovalbumin into the aortic cannula. In order to study the effects of relaxin, porcine RLX (30 ng/ml) was administered to the perfusate 30 minutes prior to the final ovalbumin injection. RLX attenuated the reduction in coronary flow and adverse inotropic and chronotropic effects caused by the ovalbumin challenge. In addition, the increases in histamine content in coronary effluent and myocardial tissue as well as mast cell degranulation were inhibited by RLX. Increased nitrite concentration, cardiac expression of iNOS and cGMP along with reduced calcium content were observed with relaxin treatment compared to controls. Based on these findings and prior work by the same authors on bronchial hyper-responsiveness and inflammatory lung injury induced by antigen challenge,[89] the mechanism by which RLX protects against cardiac anaphylaxis is via an inhibition of mast cell degranulation and preventing release of histamine and other mediators.

Relaxin and Cerebral Ischemia (Stroke)

Based on the reported properties of relaxin in the cardiovascular system and the presence of relaxin binding sites and actions on the brain, it has been hypothesized that relaxin may have a neuroprotective effect in stroke and ischemic brain disease.[108,109] Wilson and colleagues have performed preliminary studies using a rodent model of stroke.[108] Recombinant human RLX (10ng in 200 nL of saline) or 200 nL of saline was injected into the secondary somatosensory cortex of anesthetized rats. Thirty minutes after treatment, the middle cerebral artery was occluded in two areas using bipolar coagulation. After four hours, the rats were killed and brains isolated. The ratio of the infarct area to the ipsilateral hemispheric area was compared between the relaxin-treated and control groups. Proper location of injection was confirmed by analyzing brain sections. Based on four rats in each treatment group, relaxin pretreatment significantly reduced the mean infarct area/hemispheric area ratio. The investigators speculate that the possible mechanisms of the reduced tissue death with RLX treatment could be secondary to nitric oxide mediated vasodilation thereby improving perfusion and/or improved collateral circulation or alternatively via activation of estrogenic mechanisms which are neuroprotective. Recently published work does not support

the estrogenic mechanisms that were posited in this model.[110] Relaxin-induced VEGF mediated mechanisms are also postulated as being a potential contributing factor.

Relaxin and Intestinal Ischemia-Reperfusion Injury

Intestinal ischemia secondary to reduced or absent blood flow can result in endothelial injury and inflammation contributing to the pathophysiology of shock. Masini and colleagues have applied their extensive experience with cardiac ischemia-reperfusion injury to a well recognized animal model of splanchnic artery occlusion followed by reperfusion (SAO/R).[111] All rats underwent surgical splanchnic artery occlusion and the three groups were administered purified porcine RLX (30 ng/kg), inactivated RLX, or vehicle 15 minutes prior to reperfusion. In addition to reducing the drop in blood pressure caused by SAO/R, bioactive relaxin also reduced leukocyte infiltration as measured by myeloperoxidase activity and expression of endothelial cell adhesion markers in the ileum. RLX also reduced peroxidation and nitration products indicative of free radical mediated tissue injury, specifically, malondialdehyde and nitrotyrosine. Reduced markers of DNA damage and superoxide dismutase were also observed in the bioactive RLX-treated group. RLX administration was also associated with reduced ileal cell apoptosis as measured by caspase 3 and terminal deoxynucleotidyltransferase-mediated UTP end labeling. These were in contrast to the findings in SAO/R rats treated with inactive RLX or vehicle. These data suggest that, similar to myocardial ischemia-reperfusion injury, RLX may reduce reperfusion-related tissue injury in the intestinal tract.

Relaxin and Liver Transplantation—Preservation and Reperfusion

Reperfusion injury is also major problem in organ transplant. Boehnert and colleagues studied liver transplantation using isolated perfused rat livers[112] The investigators administered rhRLX in the preservation solution or with both preservation and reperfusion. rhRLX 32 ng/ml was used for reperfusion and 64 ng/ml used for preservation. The period of ischemia was 3.5 hours with one group at 20°C and the other at 4°C. Cell damage was quantified by measuring malondialdehyde and myeloperoxidase in the perfusate as well as immunohistochemical staining of the liver. In both warm and cold ischemia, relaxin treatment reduced malondialdehyde and myeloperoxidase in the perfusate as well as in the tissue staining. These exciting preliminary data suggest that relaxin may have a role in preservation as well as the reperfusion phases of organ transplantation.

Relaxin and Pulmonary Hypertension

The vasodilatory and anti-fibrotic effects of relaxin are also proposed to ameliorate pulmonary hypertension. Tozzi and colleagues utilized a hypoxia model of pulmonary hypertension in rats.[113] Two doses of rhRLX (0.24 mg/kg and 0.05 mg/kg) were administered subcutaneously for 10 days to hypoxic (10% oxygen) rats. On day 11, RLX reduced right ventricular pressure in a dose-dependent manner in these rats anesthetized with pentobarbital. In the high dose relaxin group, collagen accumulation was reduced in the main pulmonary artery compared to untreated hypoxic controls. Right ventricular pressures were not significantly decreased with RLX administration to air-breathing rats In vitro studies of cultured rat pulmonary artery fibroblast cells revealed that relaxin reduced collagen and fibronectin in TGF-β stimulated cells. These investigators suggest that relaxin may suppress fibroproliferation in hypoxia-induced pulmonary hypertension and may have therapeutic benefit in some cases of pulmonary hypertension.

Relaxin and Vascular Inflammation

Neutrophil margination within blood vessels and endothelial expression of adhesion markers can result in endothelial and vascular dysfunction as well as extravasation of neutrophils with subsequent tissue injury. Nistri and colleagues have approached this issue from the perspective of myocardial ischemia and the potential role of relaxin in ameliorating this inflammation mediated tissue injury in the heart. To this end, rat coronary artery endothelial cells primed with lipopolysaccharide were cocultured in vitro with neutrophils.[88] Pretreatment with porcine relaxin reduced the adherent neutrophils by light microscopy compared to controls pretreated with inactivated RLX.

Surface adhesion molecules, P-selectin and VCAM-1, expression by Western blot, immunohistochemistry and PCR, were reduced with relaxin pretreatment. Administration of the nitric oxide synthase inhibitor, L-NMMA, significantly attenuated these salutary effects of relaxin. Thus, a nitric oxide mediated mechanism is implicated in the reduced vascular inflammation observed with RLX pretreatment. Masini and colleagues extended these findings to human neutrophils and demonstrated that RLX could inhibit activation of isolated human neutrophils.[87] Relaxin reduced surface expression of CD11b, reduced the generation of superoxide anion, reduced the rise in intracellular calcium and reduced the release of cytoplasmic granules and chemotactic migration. These relaxin-mediated effects were blunted with L-NMMA. Thus, relaxin-mediated effects may also play a role in pregnancy and counteract the excess maternal inflammatory response seen with certain pregnancy associated disorders, such as preeeclampsia and may have therapeutic implications in other disorders associated with excessive vascular inflammation. However, not all publications are consistent with this concept.[114]

Relaxin and Preeclampsia

Preeclampsia is a pregnancy-specific disorder that occurs during the second half of gestation. This syndrome is characterized by generalized vasoconstriction and endothelial dysfunction. During active disease, the serum immunoreactive relaxin concentrations are not significantly different from those measured in normotensive, gestational-aged matched controls.[115] However, whether bioactive serum concentrations may be decreased, or whether there may be reduced numbers of vascular relaxin receptors or a defect in postreceptor signaling during preeclampsia is unknown. Furthermore, in women destined to develop preeclampsia, the status of serum relaxin concentrations during the first trimester when peak levels are reached is also unknown. It is possible that abnormally low or high serum concentrations at that time may result in deficient or exaggerated maternal renal and cardiovascular adaptations that, in turn, predispose women to develop the disease.

By virtue of its renal vasodilatory attributes, relaxin administration could improve renal plasma flow and glomerular filtration rate in women with preeclampsia. Relaxin therapy would be expected to decrease systemic vascular resistance and increase global arterial compliance, thereby augmenting cardiac output and maternal organ perfusion. There is limited information indicating that relaxin may also be a uterine vasodilator.[52] Perhaps relaxin could enhance uteroplacental blood flow by dilating unremodelled spiral arteries containing vascular smooth muscle, thereby improving oxygenation of the intervillous space and attenuating placental expression of hypoxia-inducible transcription factors and their regulated genes such as the anti-angiogenic, soluble fms-like tyrosine kinase and soluble endoglin.[116-118] Because relaxin is primarily an arterial vasodilator, preload is not compromised and thus, cardiac output is reciprocally increased, such that undue hypotension should not be a limitation of relaxin therapy. Rather, blood pressure can be reduced using the standard anti-hypertensive agents as necessary for maternal health concerns.

One question is whether more relaxin is better in preeclampsia. As mentioned, immunoreactive serum concentrations are comparable in preeclamptic and normotensive controls, but for both, they are only ~50% of the peak levels observed in the first trimester of normal pregnancies. Ultimately, the answer to this question can only be determined by clinical investigation. Another question is whether the vasodilatory properties of relaxin that ultimately depend on endothelial NO production (at least in health) are preserved in the face of the "endothelial dysfunction" that accompanies preeclampsia. In partial answer to this question, relaxin is fully active in spontaneously hypertensive rats and angiotensin II-infused rats, two animal models of hypertension associated with endothelial dysfunction.[9]

Last, based on both circumstantial evidence and preliminary experiments from our laboratory, we hypothesize that vascular endothelial growth factor (VEGF) is an intermediary molecule in the relaxin vasodilatory pathway positioned between the relaxin receptor and gelatinase. On the one hand, circulating anti-angiogenic molecules such as sFlt-1 may impair the relaxin vasodilatory pathway, thereby contributing to the pathogenesis of preeclampsia. On the other, relaxin supplementation may restore endothelial cell health in the disease by enhancing local production

of VEGF within the arterial wall, thereby partly or wholly neutralizing the deleterious effects of circulating anti-angiogenic factors. Finally, the anti-inflammatory properties of relaxin as reviewed above (Relaxin and vascular inflammation) may be beneficial in the setting of preeclampsia.

Part 3. Controversies, Unresolved Issues and Future Directions

Over the past decade, relaxin has emerged as a hormone with multiple vascular actions. However, there are a number of controversies or unresolved issues and some of the more glaring ones are summarized below. Hopefully other laboratories will weigh in on these controversies with additional data in the near future.

1. There are two, very different proposals to explain the vasodilatory action of relaxin. One advanced by Bani and colleagues implicates the induction of iNOS and the other by Conrad and coworkers implicates the activation of eNOS (supra vide).
2. The discrepant findings of Dschietzig and colleagues[35] and Kerchner et al[34] on whether Rlx upregulates the endothelial ET_B receptor remains unresolved.
3. Li and colleagues[119] recently claimed that chronic relaxin administration to rats did not reduce the myogenic reactivity of isolated mesenteric arteries and they attributed the apparent reductions observed by Novak and coworkers[27] to increased passive compliance. However, other explanations for the different results arising from the two laboratories are possible. First, Li et al investigated *myogenic tone* in which the main independent variable is the presence or absence of extracellular calcium (at any given pressure), while Novak and colleagues examined *myogenic reactivity* where the main independent variable is change in intraluminal pressure (in the presence of calcium). Therefore, the two studies may not be directly comparable. Second, Li and colleagues used small mesenteric arteries, while Novak and coworkers have focused on small renal arteries. There may be differences secondary to the anatomical origin of arteries. Third, the myogenic reactivity of small renal arteries isolated from midterm pregnant rats is reduced, yet passive compliance remains unchanged at this stage of pregnancy[10,11]. This reduction in myogenic reactivity is due to relaxin, because it was found to be reversed in small renal arteries isolated from midterm pregnant rats that received relaxin neutralizing antibodies in vivo.[5] Therefore, the reduction in myogenic reactivity due to relaxin is found to occur in the absence of any increase in passive compliance. Fourth, the reduction in myogenic reactivity of small renal arteries isolated from nonpregnant rats chronically administered rhRlx is reversed by short-term treatment with NO synthase inhibitors, ET_B receptor antagonists and MMP inhibitors.[27,36] It is highly unlikely that the increase in passive compliance which reflects a remodeling of the vascular wall will be reversed by these inhibitors in such a short period of time (30 min). A similar conclusion can be drawn for the recent investigations on arterial function in wild-type and relaxin knock-out mice in which short-term, 30 min incubations with L-arginine were used.[72]
4. There is not unanimity among the studies on whether serum relaxin is significantly increased in congestive heart failure.[73,101] Furthermore, despite using the same relaxin assay, serum relaxin concentrations were not uniform between the studies. The latter issue points to a general deficiency in the field, i.e., the availability of validated, reliable and sensitive, commercial assays for measuring human relaxin.

In view of the numerous and generally beneficial actions of relaxin in the vasculature learned mainly from animal investigations, there is tremendous potential for therapeutics. Indeed, the therapeutic potential of relaxin has been tested and validated in various animal models of human disease. Future investigations need to establish whether these beneficial vascular actions of relaxin translate to humans.

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