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G-Protein-coupled receptors as potential drug candidates in preeclampsia: targeting the relaxin/insulin-like family peptide receptor 1 for treatment and prevention

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BACKGROUND: Important roles for G-protein-coupled receptors (GPCRs) have been identified in the maternal physiological adaptations to pregnancy and in the pathogenesis of preeclampsia. On this basis, GPCRs are potential therapeutic targets for preeclampsia.

OBJECTIVES AND RATIONALE: In this review, vasopressin and apelin are initially considered in this context before the focus on the hormone relaxin and its cognate receptor, the relaxin/insulin-like family peptide receptor 1 (RXFP1). Based on both compelling scientific

rationale and a promising safety profile, the relaxin ligand–receptor system is comprehensively evaluated as a potential therapeutic endpoint in preeclampsia.

SEARCH METHODS: The published literature relating to the topic was searched through January 2016 using PubMed.

OUTCOMES: Relaxin is a peptide hormone secreted by the corpus luteum; it circulates in the luteal phase and during pregnancy. Activation of RXFP1 is vasodilatory; thus, relaxin supplementation is expected to at least partly restore the fundamental vasodilatory changes of normal pregnancy, thereby alleviating maternal organ hypoperfusion, which is a major pathogenic manifestation of severe preeclampsia. Specifically, by exploiting its pleiotropic hemodynamic attributes in preeclampsia, relaxin administration is predicted to (i) reverse robust arterial myogenic constriction; (ii) blunt systemic and renal vasoconstriction in response to activation of the angiotensin II receptor, type 1; (iii) mollify the action of endogenous vasoconstrictors on uterine spiral arteries with failed remodeling and retained smooth muscle; (iv) increase arterial compliance; (v) enhance insulin-mediated glucose disposal by promoting skeletal muscle vasodilation and (vi) mobilize and activate bone marrow-derived angiogenic progenitor cells, thereby repairing injured endothelium and improving maternal vascularity in organs such as breast, uterus, pancreas, skin and fat. By exploiting its pleiotropic molecular attributes in preeclampsia, relaxin supplementation is expected to (i) enhance endothelial nitric oxide synthesis and bioactivity, as well as directly reduce vascular smooth muscle cytosolic calcium, thus promoting vasodilation; (ii) improve the local angiogenic balance by augmenting arterial vascular endothelial and placental growth factor (VEGF and PLGF) activities; (iii) ameliorate vascular inflammation; (iv) enhance placental peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PCG1α) expression, and hence, peroxisome proliferator-activated receptor gamma (PPAR-γ) activity and (v) confer cytotrophoblast and endothelial cytoprotection. Insofar as impaired endometrial maturation (decidualization) predisposes to the development of preeclampsia, relaxin administration in the late secretory phase and during early pregnancy would be anticipated to improve decidualization, and hence trophoblast invasion and spiral artery remodeling, thereby reducing the risk of preeclampsia. Relaxin has a favorable safety profile both in the non-pregnant condition and during pregnancy.

WIDER IMPLICATIONS: There is a strong scientific rationale for RXFP1 activation in severe preeclampsia by administration of relaxin, relaxin analogs or small molecule mimetics, in order to mollify the disease pathogenesis for safe prolongation of pregnancy, thus allowing time for more complete fetal maturation, which is a primary therapeutic endpoint in treating the disease. In light of recent data implicating deficient or defective decidualization as a potential etiological factor in preeclampsia and the capacity of relaxin to promote endometrial maturation, the prophylactic application of relaxin to reduce the risk of preeclampsia is a plausible therapeutic approach to consider. Finally, given its pleiotropic and beneficial attributes particularly in the cardiovascular system, relaxin, although traditionally considered as a 'pregnancy' hormone, is likely to prove salutary for several disease indications in the non-pregnant population.

Key words: arginine vasopressin / apelin / endothelium / hemodynamics / insulin resistance / cell death / nitric oxide / angiogenic growth factors / PPAR / placenta

Introduction

Severe preeclampsia can be a volatile, dangerous, and potentially life-threatening syndrome. The disease afflicts 3–5% of pregnancies, typically manifesting during the third trimester, and is a major cause of maternal, fetal and neonatal, morbidity and mortality (Duley, 2003; Backes *et al.*, 2011; Ghulmiyyah and Sibai, 2012). Globally, ~7 million women annually are afflicted by preeclampsia, which is diagnosed by new onset hypertension after 20 gestational weeks accompanied by one or more of the following features: proteinuria, thrombocytopenia, renal or hepatic insufficiency, pulmonary edema, cerebral or visual symptoms (Hypertension in pregnancy, 2013; Kuklina *et al.*, 2009). In developed countries, preeclampsia often leads to iatrogenic premature delivery that can cause major morbidity in the neonate immediately after delivery and long term. In the developing world where (timely) access to health care may be lacking, preeclampsia can result in maternal, fetal and neonatal death (Duley, 2003; Backes *et al.*, 2011; Ghulmiyyah and Sibai, 2012). Of equal or perhaps even of greater concern is that women and children who survive preeclampsia are at greater risk for developing future adverse cardiovascular events (Roberts *et al.*, 2003; Sibai *et al.*, 2005; Anderson, 2007; Bellamy *et al.*, 2007; Gluckman *et al.*, 2008; Vikse *et al.*, 2008; Williams, 2011; Davis *et al.*, 2012). Whether preeclampsia itself inflicts long-term, residual vascular damage in the mother is unclear (Epstein, 1964; Levine *et al.*, 2009), but if so, it is not inconceivable that amelioration of the disease during pregnancy could mitigate the increased risk of remote cardiovascular disease. Hence, effective treatments for preeclampsia are sought not only to alleviate morbidity, mortality and iatrogenic premature delivery in the perinatal period, but because they may also lower remote cardiovascular disease risk in affected women and their children. In addition, low birth weight is a frequent neonatal outcome of preeclampsia (Rich-Edwards *et al.*, 2015) and is independently associated with elevated occurrence of hypertension and ischemic heart disease (among other pathologies) during adulthood (Barker *et al.*, 1989, 1990). Disappointingly, despite first being recognized over 2000 years ago, therapies for preeclampsia that counteract the pathogenic mechanisms are currently lacking. Rather, 'palliative' measures are imposed to manage blood pressure and prevent seizures, and delivery remains the only known cure. Nor are there prophylactic strategies available, although low dose aspirin may have a small protective effect in women at elevated risk of developing preeclampsia (Henderson *et al.*, 2014).

Development of therapeutics specific for preeclampsia has been hampered by several factors. First, an insufficient understanding of the pathophysiology, and consequently, a lack of known pathogenic

targets has precluded their development. However, our understanding of pathophysiology has improved over the past decade, insofar as circulating molecules of placental origin and maternal constitutional factors that contribute or predispose to disease manifestations have been identified (Roberts *et al.*, 2003; Young *et al.*, 2010; Redman *et al.*, 2012). Second, preeclampsia appears to be a uniquely human affliction, which has reduced enthusiasm for underwriting research proposals designed to test potential therapeutics in animal models of the disease (Conrad, 1990; Granger *et al.*, 2014). Third, confined vision and short-range goals, low risk tolerance and funding pay lines have conspired to assign low priority to grant applications in pursuit of novel therapeutic candidates. Last, the pharmaceutical industry has traditionally exercised risk aversion for developing therapeutics in pregnancy-related diseases.

Despite these handicaps, several potential therapeutic targets including the G-protein-coupled receptor (GPCR) relaxin/insulin-like family peptide receptor, RXFP1, and its cognate ligand, relaxin, were showcased in a first-ever symposium titled 'Novel Therapies for Preeclampsia' at the *International Society for the Study of Hypertension in Pregnancy Meeting* October 2014. The main goal of this review is to extend the (necessarily abridged) relaxin presentation at this meeting, particularly in the context of recent findings in the field, with the goal of comprehensively evaluating the scientific rationale for considering relaxin or relaxin mimetics as potential therapeutics in preeclampsia. To this end, cardiovascular function in uncomplicated pregnancy and preeclampsia will first be discussed. Next, several candidate GPCRs will be reviewed as potential drug targets in the disease. Last, a comprehensive appraisal of RXFP1, arguably one of the most promising therapeutic targets in preeclampsia, will be presented.

Methods

PubMed searches through January 2016 were conducted using the keywords: adrenomedullin-2/intermedin and amylin, ligands and receptors; apelin and apelin receptor, arginine vasopressin and vasopressin receptor, vasopressinase, copeptin, and oxytocin in the context of pregnancy, preeclampsia and placenta. The relaxin receptor and relaxin, as well as relaxin/insulin-like family peptides Insl 3–6 were also searched, each as standalone keywords or phrases. Not all of the topics yielded sufficient literature to be included. This work critically evaluates the apelin, vasopressin and relaxin ligand–receptor systems as potential therapeutic targets in preeclampsia.

Cardiovascular function in normal pregnancy and preeclampsia

As summarized in Table I, normal pregnancy is associated with profound vasodilation of the maternal renal and systemic circulations. Reduced renal and systemic vascular resistances lead to marked increases in renal blood flow, glomerular filtration rate and cardiac output (Conrad, 2011). These physiological changes commence during the luteal phase of the menstrual cycle, becoming more pronounced in the first trimester and thereafter (Jeyabalan and Conrad, 2010). Two major events conspire to increase cardiac output during normal pregnancy: a decline in systemic vascular resistance or cardiac afterload (to which renal vasodilation majorly contributes) and a concurrent increase of venous return to the heart or cardiac preload; the latter at least partly results from mobilization of the large reservoir of blood located in the splanchnic circulation. This mobilization is made possible by reduced hepatic vascular resistance, mainly regulated in the sinusoids of the microvasculature, as reflected by augmentation of portal venous and hepatic artery blood flow during normal pregnancy (Clapp *et al.*, 2000; Nakai *et al.*, 2002; Vollmar and Menger, 2009; Mandic-Markovic *et al.*, 2014). Increased splanchnic venous tone is another likely mechanism contributing to mobilization of blood from the splanchnic circulation. Particularly pronounced in the second half of pregnancy, plasma volume expands dramatically, further augmenting cardiac preload (Dechend *et al.*, 2015). Finally, uterine blood flow also increases markedly, especially in the latter half of gestation during the rapid growth phase of the fetus and placenta (Dickey and Hower, 1995).

There are additional changes in the maternal circulation consistent with, or contributing to, the profound vasodilatory state (Table I). These include attenuated pressor responses to vasoconstrictors such as angiotensin II and norepinephrine (Gant *et al.*, 1973; Nisell *et al.*, 1985; Conrad and Colpoys, 1986; Danielson and Conrad, 1995), inhibited arterial myogenic constriction (Gandley *et al.*, 2001; Matsubara *et al.*, 2006), robust endothelial function (Sladek *et al.*, 1997) and increased global arterial compliance, the latter being required to maintain ventricular–arterial coupling in the face of the dramatic decline in systemic vascular resistance (Poppas *et al.*, 1997; Debrah *et al.*, 2006). Another intriguing pregnancy adaptation is an increased number of circulating maternal bone marrow-derived angiogenic progenitor cells (Gammill *et al.*, 2007). Although the role of these angiogenic progenitor cells in pregnancy remains unexplored, we hypothesized that they abet maternal angiogenesis and vasculogenesis in organs such as breast, uterus, adipose, skin and pancreas, further contributing to reduced systemic vascular resistance and increased global arterial compliance (Segal *et al.*, 2012), among other physiological roles.

During active disease, the aforementioned maternal adaptations to normal pregnancy are impaired in women with preeclampsia (Table I). The typical vasodilatory responses are either reduced or completely absent resembling the non-pregnant state, and in many cases, profound vasoconstriction ensues. Thus, systemic and renal vascular resistances are elevated relative to normal pregnancy with corresponding reductions in cardiac output, renal blood flow and glomerular filtration rate (Conrad and Davison, 2014; Hibbard *et al.*, 2015). In one study, evidence for increased hepatic vascular resistance was reported in preeclampsia with or without HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome (Oosterhof *et al.*, 1994), while in another, reduced hepatic arterial and portal venous blood flow was observed in preeclampsia, but only when accompanied by HELLP (Kawabata *et al.*, 2006). Thus, changes in the hepatic microcirculation likely impede mobilization of blood from the splanchnic reservoir; this deficit, in concert with the well-known reduction in plasma volume during preeclampsia (Dechend *et al.*, 2015), further compromises cardiac preload and output. Last, uterine blood flow is frequently depressed in preeclampsia contributing to fetal growth restriction (Velauthar *et al.*, 2014).

Relative to normal pregnancy, in preeclampsia the pressor responses to vasoconstrictors such as angiotensin II and

norepinephrine are enhanced (Gant *et al.*, 1973; Nisell *et al.*, 1985), global arterial compliance is reduced (Hibbard *et al.*, 2015), endothelial structure and function are compromised (Davidge *et al.*, 2015), and there are fewer circulating maternal, bone marrow-derived angiogenic progenitor cells (Gammill *et al.*, 2007). Consistent with the last observation is reduced capillary density of the nail beds in preeclamptic women (Hasan *et al.*, 2002). A major pathogenic mechanism leading to endothelial dysfunction is believed to be the angiogenic imbalance created by excess production and release into the maternal circulation of soluble vascular endothelial growth factor receptor-1 (sVEGF-R1) among other factors mainly derived from the stressed placenta (Maynard *et al.*, 2003; Venkatesha *et al.*, 2006). Compared to the metabolic adaptations observed in normal pregnancy, there is further impairment of glucose disposal and insulin resistance in women with preeclampsia (Table I) (Catalano, 2010). A contributing factor is likely to be pathological compromise of insulin-mediated increases in skeletal muscle blood flow, an event mediated through endothelial-derived nitric oxide (Steinberg *et al.*, 1994). Finally, based on a limited number of experiments, myogenic constriction of subcutaneous arteries isolated from women with preeclampsia is robust rather than inhibited as in normal pregnancy, thus resembling the non-pregnant condition (Debrah, Novak and Conrad, unpublished preliminary data; Fig. 1; Table I). In essence, endothelial dysfunction can explain many of the disease manifestations of preeclampsia including hypertension, proteinuria and excessive edema.

Table I Pathophysiological features of preeclampsia.

Normal pregnancy	Preeclampsia
Systemic and renal vasodilation	Systemic and renal vasoconstriction
↓ Sensitivity to vasoconstrictors	↑ Sensitivity to vasoconstrictors
↑ Arterial compliance	↓ Arterial compliance
↓ Myogenic constriction	↑ Myogenic constriction
↑ Uterine blood flow	↓ Uterine blood flow
↑ Circulating bone marrow-derived angiogenic progenitor cells	↓ Circulating bone marrow-derived angiogenic progenitor cells
Robust endothelial function	Endothelial dysfunction
↑ Insulin resistance	↑↑ Insulin resistance

↑ increased, ↓ decreased: normal pregnancy relative to the non-pregnant condition or preeclampsia relative to normal pregnancy. See text for further details and references.

Potential role of GPCRs as therapeutic targets in preeclampsia

Because a large proportion of the GPCR target pharmaceuticals on the market today actually represent only a small number of the GPCRs that could be exploited for medicinal use, the scope of this

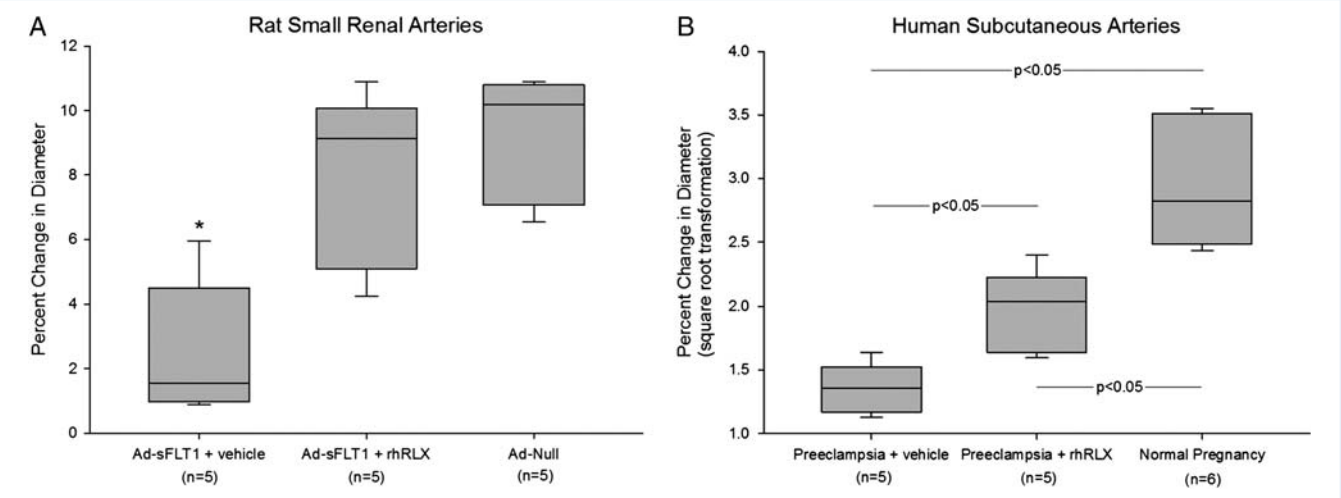

Figure 1 Normal pregnancy phenotype of inhibited myogenic constriction is restored (**A**) or party restored (**B**) by incubation of arteries from pathological pregnancies with recombinant human relaxin (rhRLX; 30 ng/ml) for 3 hours *in vitro*. Myogenic constriction is expressed as the percentage change of internal diameter in response to a step increase of intraluminal pressure from 60 to 80 mmHg after reaching a new steady state (~5 minutes). A larger percent change in diameter corresponds with inhibition of myogenic constriction. Box depicts median, first and third quartiles, and whiskers indicate the highest and lowest data points. (A) Small renal arteries were harvested from the sVEGF-R1 (or sFLT1) rat model of preeclampsia (Maynard *et al.*, 2003) (kidneys kindly provided by SA Karumanchi, Harvard Medical School) under IACUC approval from the Magee-Womens Research Institute, University of Pittsburgh. Ad-sFLT1, adenovirus containing the sFLT1 construct; Ad-Null, adenovirus without the sFLT1 construct. N = number of rats. $P < 0.001$ by one way-ANOVA. *$P < 0.05$ vs Ad-sFLT1 + rhRLX and Ad-Null by Student Newman Keuls (SNK) *post hoc* test. (B) Small subcutaneous arteries were dissected from adipose tissue obtained from the Caesarean section site under IRB approval from the Magee-Womens Research Institute, University of Pittsburgh. N = number of subjects. In order to pass the Normality and Equal Variance Tests, the data were subjected to square root transformation. $P < 0.001$ by one way-ANOVA. *P*-values in the figure were derived from the SNK *post hoc* test. (Debrah, Novak and Conrad, preliminary unpublished data). See text for abbreviations, further details and references.

review will encompass these seven-transmembrane-domain receptors. GPCRs and their ligands with cardiovascular actions have been evaluated in normal pregnancy and preeclampsia. McGuane and Conrad recently reviewed several GPCRs in the context of possible drug development for preeclampsia including calcitonin receptor-like receptor/RAMP1, calcitonin receptor-like receptor/RAMP2, angiotensin 1 and 2 receptors, and the MAS receptor (McGuane and Conrad, 2012). Endothelin-1 and its corresponding GPCR, the endothelin A receptor, were discussed by Granger and colleagues at the 2014 ISSHP Meeting symposium on novel therapies for preeclampsia. The reader is referred to a recent review of the therapeutic potential of endothelin A (ET_A) receptor antagonists by this research group (Sasser *et al.*, 2015). Thus, these GPCRs and their cognate ligands will not be further considered in this review, rather two others recently emerging receptors in the preeclampsia field will be critically evaluated, namely, the arginine vasopressin (AVP) and apelin (APJ) receptors. Although other GPCRs and cognate ligands might be scrutinized, including adrenomedullin-2/intermedin, amylin and other members of the relaxin/insulin-like family peptides Insl 3–6, there is little or no information to date describing their potential involvement in preeclampsia. The concept of RXFP1 as a potential therapeutic target was previously advanced (Davison *et al.*, 2004; Dechend and Luft, 2008; Unemori *et al.*, 2009; McGuane and Conrad, 2012). However, the scientific rationale for relaxin or relaxin mimetics as potential therapeutics in preeclampsia has not been comprehensively laid out especially in the context of recent advances made in relaxin research. Moreover, irrespective of the outcome of the Phase 3B Clinical Trial of recombinant human relaxin (Serelaxin) in acute heart failure initiated 2014 (ClinicalTrials.gov Identifier: NCT02064868), the timing is right to set the stage for preeclampsia as one of the next disease targets for relaxin. To this end, compelling scientific rationale enters prominently in the decision-making by the pharmaceutical industry to embark upon clinical trials of relaxin in preeclampsia, and by clinicians and their patients to participate in these trials.

Arginine vasopressin receptors

Arginine vasopressin is a 9-amino acid peptide produced by the hypothalamus and released from the posterior pituitary in response to increases in plasma osmolality or reductions in blood pressure or volume (Valtin and Schafer, 1995). The sensitivity of the former mechanism is exquisite requiring only 1 to 2 mOsm/Kg H_2O elevation in plasma osmolality, while the latter is only triggered by relatively large ~10% declines, but the gain is considerably higher, yielding marked increases in plasma AVP. AVP then acts on kidney tubules through the V2 receptor to promote water reabsorption, and on blood vessels through the V1 receptor to cause vasoconstriction, thereby primarily restoring water homeostasis but also blood volume and pressure. Obviously, thirst is the other essential component of this homeostatic system.

A reduction in the osmotic threshold for both release of AVP and stimulation of thirst transpires in the late luteal phase continuing during early pregnancy (Conrad and Karumanchi, 2013). Relaxin may be the mediator of these osmoregulatory changes in pregnancy (Weisinger *et al.*, 1993; Danielson *et al.*, 1999; Novak *et al.*, 2001). Thus, AVP circulates in concentrations comparable to the non-pregnant condition, but at a plasma osmolality of ~10 mOsm/Kg H_2O lower; otherwise, pregnant women concentrate and dilute their urine appropriately around this lower set point of plasma osmolality (Conrad and Karumanchi, 2013). Rarely, pregnant women may experience transient diabetes insipidus secondary to inappropriately high circulating vasopressinase emanating from the placenta (Barron *et al.*, 1984; Durr *et al.*, 1987). This syndrome can occur in the setting of hepatic dysfunction associated with acute fatty liver, HELLP syndrome and preeclampsia, because vasopressinase is normally cleared by the liver (Durr and Lindheimer, 1996).

The pre-pro-vasopressin precursor in the hypothalamus consists of a signal peptide, AVP, neurophysin II and copeptin. Like neurophysin II, copeptin may be involved in the intracellular processing of AVP, but otherwise this 39 amino acid peptide (~5 kDa) has no known function. Neurophysin II and copeptin are released from the posterior pituitary in a 1:1 molar ratio with AVP (Morgenthaler *et al.*, 2006). There are technical challenges to measuring plasma AVP including the necessity for radioimmunoassay (RIA) due to its small molecular weight (1084 Da) and low circulating concentration (1–10 pM), and the importance for prior extraction of plasma before RIA (Robertson *et al.*, 1973). In addition, AVP binds to platelets with high avidity (Preibisz *et al.*, 1983), its immunoreactivity decreases rapidly *ex vivo* even at −20°C (Robertson *et al.*, 1973), and the hormone has a short half-life *in vivo* particularly in pregnancy due to circulating leucyl/cystinyl aminopeptidases (vasopressinase or oxytocinase) emanating from the placenta. Because copeptin is released in equimolar quantities from the posterior pituitary, it was evaluated as a possible surrogate for AVP. Copeptin circulates in sufficiently high concentrations to be assayed by sensitive sandwich immunoassay and is stable *ex vivo* (although it also has a short half-life *in vivo*). Thus, Morgenthaler *et al.* (2006) investigated copeptin as a surrogate measurement for AVP showing an excellent correlation with AVP in normal healthy subjects and patients with sepsis. Balanescu *et al.* (2011) subsequently demonstrated strong correlations between copeptin and AVP in healthy volunteers subjected to water load and hypertonic saline challenges.

A potential pathogenic role of arginine vasopressin in preeclampsia was proposed based on elevated circulating concentrations of copeptin. Most (Zulfikaroglu *et al.*, 2011; Santillan *et al.*, 2014; Yeung *et al.*, 2014; Tuten *et al.*, 2015), but not all (Birdir *et al.*, 2015) groups reported elevated circulating copeptin levels before and/or during the clinical manifestations of the disease. In one study, the increase in copeptin concentrations measured at 16 weeks of gestation was specific for preeclampsia, not being observed in women who developed gestational hypertension (without proteinuria), gestational diabetes mellitus or preterm birth in the absence of preeclampsia (Yeung *et al.*, 2014). Santillan *et al.* (2014) reported that increases in copeptin early in gestation were predictive of preeclampsia, and chronic administration of AVP to pregnant, but not non-pregnant mice significantly increased systolic blood pressure by 5–10 mmHg and induced proteinuria, glomerular endotheliosis and fetal growth restriction, all hallmarks of preeclampsia, thus providing proof of principle that AVP may be a pathogenic agent in the disease.

Although the potential role for AVP in the pathogenesis of preeclampsia is intriguing, there are perhaps a few cautionary points that should be made. First, although Morgenthaler *et al.* (2006)

meticulously validated the copeptin sandwich immunoassay for plasma from non-pregnant subjects, apparently copeptin immunoassays have not been validated using plasma from normal pregnant and pre-eclamptic women as matrices in the assay. Second, as discussed above, Morgenthathler and coworkers also demonstrated a significant correlation between copeptin and AVP in healthy non-pregnant subjects and patients with sepsis, and this correlation was subsequently corroborated by Balanescu and colleagues. But, so far, this relationship has apparently not been established for normal pregnancy and preeclampsia. Elevated copeptin in preeclamptic women could conceivably be secondary to other reasons such as decreased hepatic or renal clearance. In an attempt to rule out renal clearance as a confounder, Santillan *et al.* (2014) showed that plasma cystatin C was comparable in the women who developed preeclampsia or experienced a normal pregnancy outcome. But there is considerable controversy about whether cystatin C is a reliable marker of renal function in pregnancy (Saxena *et al.*, 2012), perhaps in part due to placental production (Kristensen *et al.*, 2007). Because copeptin is only ~5 kDa, it likely undergoes glomerular filtration, which is frequently compromised in preeclampsia.

The measurement of AVP *per se* is really critical to the hypothesis. Indeed, many investigators have successfully measured AVP in non-pregnant subjects (Robertson *et al.*, 1973; Balanescu *et al.*, 2011; El-Farhan *et al.*, 2013), and by taking appropriate steps to inactivate vasopressinase, AVP measurements in pregnancy have also been reliable (Davison *et al.*, 1984). Inconsistent with the hypothesis is that, to date, not a single publication has demonstrated elevated AVP or reduced plasma osmolality in preeclampsia relative to normal pregnancy (MacGillivray *et al.*, 1976; Pedersen *et al.*, 1985; van der Post *et al.*, 1993; Gordge *et al.*, 1994; Eguchi *et al.*, 1996), except for the occasional report of severe hyponatremia in which AVP was not measured, but presumed to be elevated due to volume contraction and consequent non-osmotic release (Wilson and Shutt, 2007).

Nevertheless, the mouse model developed by Santillan *et al.* (2014) is promising particularly because the preeclampsia-like changes elicited by AVP administration were restricted to pregnancy and not observed in the non-pregnant animals. However, measurements of arterial pressure by tail-cuff should be confirmed using telemetry-based approaches in conscious, unstressed mice, and based on studies in conscious rats and dogs (Gellai *et al.*, 1984; Brown *et al.*, 1986; Conrad *et al.*, 1993), chronic infusion of AVP even at low doses is likely to exert profound changes in renal and osmoregulatory function, which were not examined in the study by Santillan and coworkers. Thus, the value of this animal model may be obfuscated by markedly altered physiology created by the AVP infusion and not typically observed in preeclampsia. Moreover, a clear mechanism linking AVP to preeclampsia symptoms was not established. Thus, although provocative, this animal model requires further study.

Previously, it was hypothesized that vasopressinase might be the link between AVP and preeclampsia (Krege and Katz, 1990). That is, hydrolysis of AVP at the Cys–Tyr bond by vasopressinase precludes interaction with the V2 receptor by breaking the ring structure, while preserving the N terminus required for V1 activity. Earlier studies in rats, dogs and humans demonstrate that AVP is vasodilatory in the setting of V1 blockade, illustrating the opposing vascular actions of these two receptor subtypes (Glanzer *et al.*, 1982; Liard, 1986; Walker, 1986; Naitoh *et al.*, 1993; Cooke *et al.*, 2001). Thus, it is not inconceivable that unopposed V1 activation by 'vasopressinase altered vasopressin' could promote vasoconstriction, platelet aggregation and endothelial dysfunction. One argument in favor of this hypothesis is that transient diabetes insipidus of pregnancy believed to be secondary to excessive placental production of vasopressinase can occur in the setting of preeclampsia, but this is an association, and as such, does not discern cause from consequence. Because vasopressinase is cleared by the liver and hepatic function can be compromised in preeclampsia, then preeclampsia is the likely cause of elevated vasopressinase and diabetes insipidus, not vice versa, although a feed forward mechanism remains a possibility. If circulating vasopressinase is elevated before the onset of disease manifestations, then a causal relationship might be inferred. However, vasopressinase was comparable in the first and second trimesters between women who developed preeclampsia or experienced a normal pregnancy outcome, and it was actually decreased during the third trimester in preeclamptic women (Santillan *et al.*, 2014). Finally, mice do not have placental vasopressinase (Pham *et al.*, 2009), and as such, one cannot invoke 'vasopressinase altered vasopressin' activation of V1 receptors in the mouse study by Santillan *et al.* (2014).

In conclusion, although both intriguing and provocative, whether AVP or a degradation product has a pathogenic role in preeclampsia requires further investigation. As a logical extension of this hypothesis, perhaps oxytocin should be considered in the context of its own receptor on vascular smooth muscle or even in relation to the V1 receptor despite ~30X less affinity than AVP (Holmes *et al.*, 2003). Circulating oxytocin concentrations increase progressively during normal human pregnancy and manifest episodic peaks during labor and lactation (Dawood *et al.*, 1979, 1981; Risberg *et al.*, 2009), the latter perhaps affording a pathogenic link to *postpartum* pre-eclampsia. However, like AVP, circulating oxytocin does not appear to be elevated in preeclampsia either (Risberg *et al.*, 2009). Finally, because copeptin has no known function other than a potential role in processing of the AVP pre-pro-hormone before secretion, perhaps this peptide warrants investigation rather than or in addition to AVP and oxytocin.

Apelin receptor

The 77-amino acid pre-pro-apelin dimer is expressed in several tissue- and cell-types including heart and endothelium, where it is subsequently cleaved by endopeptidases into apelin-36, -17, -13 and after post-translational modification, pyroglumatamate (pyr1)apelin-13. These peptides are agonists of the apelin G protein-coupled-receptor, APJ (Pitkin *et al.*, 2010). APJ also has widespread tissue and cell distribution including endothelium and vascular smooth muscle (Barnes *et al.*, 2010). In many endothelium-intact blood vessels, apelin stimulates NO production leading to vascular smooth muscle relaxation and vasodilation, whereas in endothelium-denuded vessels, vasoconstriction ensues. Apelin inhibits angiotensin II-induced elevated cytosolic calcium and vasoconstriction contributing to its overall vasodilatory action (Hus-Citharel *et al.*, 2008). Apelin is expressed in the supraoptic and paraventricular nuclei of the hypothalamus where it decreases AVP release. Water loading and fluid restriction lower and raise plasma AVP, respectively; the same maneuvers raise and lower plasma apelin suggesting a counter-regulatory role for apelin in

water homeostasis (Pitkin *et al.*, 2010). Apelin is also an adipokine that may abet insulin action (Boucher *et al.*, 2005).

Using immunohistochemistry, Cobellis *et al.* (2007) reported increased expression of apelin and APJ in many cellular compartments of the placenta from women with preeclampsia. However, subsequent studies failed to corroborate these findings. In one study, apelin and APJ mRNA and protein were reduced in preeclamptic placentas as determined by RT-PCR and western blot/immunohistochemistry, respectively (Inuzuka *et al.*, 2013), and in another, placental apelin was assessed by radioimmunoassay and also found to be decreased (Yamaleyeva *et al.*, 2015). In the same report, increased ACE2 expression was observed in the preeclamptic placenta, which could explain reduced apelin, because apelin is metabolized by ACE2; also, (pyr1)apelin-13 was demonstrated to be the major isoform in placenta (Yamaleyeva *et al.*, 2015).

The reports on circulating apelin in preeclampsia are not entirely consistent either, although the majority report an increase. Bortoff *et al.* (2012) observed a decrease in circulating apelin in preeclamptic women relative to normal pregnancy, while three groups reported increases in circulating apelin during early and late onset, as well as mild and severe, preeclampsia (Simsek *et al.*, 2012; Inuzuka *et al.*, 2013; Kucur *et al.*, 2014). The higher circulating levels may serve a compensatory role to counteract systemic vasoconstriction in preeclampsia. On the surface, lower expression of apelin in preeclamptic placentas appears to be incongruous with higher concentrations measured in the blood, but clearly, other tissues may contribute to circulating levels and differences in metabolic clearance could also come into play. On balance, more investigations of apelin and its receptor in preeclampsia are needed, but in light of its vasodilatory and other potentially beneficial properties, the apelin/APJ receptor system may be a potential therapeutic target in preeclampsia.

Relaxin/insulin-like family peptide receptor 1

Role of relaxin in the cardiovascular adaptations to pregnancy

In 1926, relaxin was discovered by Frederick Hisaw to be a factor circulating in the blood of late pregnant rabbits and guinea pigs. When the blood was injected into virgin guinea pigs during early post-estrus, it produced relaxation of the pubic ligament (Hisaw, 1926; Sherwood, 1994). Relaxin was later identified as a ~6 kDa peptide, and more recently the relaxin receptor was revealed to be a leucine-rich repeat GPCR (relaxin/insulin-like family peptide receptor, RXFP1) (Hsu *et al.*, 2002; Bathgate *et al.*, 2013). The hormone emanates from the corpus luteum circulating during the luteal phase and during pregnancy in many species (Sherwood, 1994). In women, relaxin reaches serum concentrations of ~0.1 ng/ml on Days 7–12 post luteinizing hormone-surge in a non-conceptive cycle, and in a conceptive cycle, relaxin rises to ~1 ng/ml during the first trimester, falling to intermediate values thereafter for the duration of pregnancy (Szlachter *et al.*, 1982; Stewart *et al.*, 1990). Relaxin has traditionally been considered to be a reproductive hormone modifying the reproductive tract in some, but not all species (Sherwood, 1994). Later, relaxin was found to exert actions in the circulation (Hisaw *et al.*, 1967; St-Louis and Massicotte, 1985; Massicotte *et al.*, 1989; Bani-Sacchi *et al.*, 1995; Danielson *et al.*, 1999), and within the last decade or two, has become firmly ensconced in the cardiovascular field.

The role of relaxin in the maternal systemic and renal adaptations to pregnancy was extensively explored in the conscious gravid rat model, because this species undergoes gestational circulatory changes similar to women (Conrad and Davison, 2014). Specifically, neutralization or elimination of circulating relaxin with specific antibodies or by ovariectomy, respectively, inhibited the increases of cardiac output, global arterial compliance, renal blood flow and glomerular filtration rate, as well as the decreases in systemic and renal vascular resistances, and in plasma osmolality during midterm pregnancy (Novak *et al.*, 2001; Debrah *et al.*, 2006). Small renal arteries harvested from gravid rats with neutralized or absent circulating relaxin demonstrated robust myogenic constriction similar to the non-pregnant condition rather than the inhibited myogenic constriction typical of normal pregnancy (Novak *et al.*, 2001). Interestingly, the relaxin-neutralizing antibodies only inhibited ~50% of the gestational increase in cardiac output and global arterial compliance, and reduction in systemic vascular resistance during late gestation suggesting a role for other vasodilator(s) possibly of placental origin (Debrah, Shroff and Conrad, unpublished data). However, additional experiments are needed to explain these observations from late stage rat pregnancy.

Whether relaxin (or other corpus luteal factors) plays a similar role in the cardiovascular and renal circulations during human pregnancy is currently under investigation. To this end, a multitude of cardiovascular and renal parameters are being assessed in women who conceive with and without assisted reproductive technologies (ART). On the one hand, women conceiving through oocyte donation lack a corpus luteum, and hence, circulating relaxin, as a consequence of ovarian failure or hypothalamic-pituitary suppression, the latter utilized as part of the ART procedure (Johnson *et al.*, 1991). Similarly, many women who undergo autologous frozen embryo transfer frequently do so in a medicated cycle involving hypothalamic-pituitary suppression. On the other hand, women undergoing ovarian stimulation, IVF and fresh embryo transfer have multiple corpora lutea, and thus, elevated levels of circulating relaxin (Mushayandebvu *et al.*, 1998). Relative to spontaneous conceptions, we anticipate subdued changes in the maternal circulation ('hypodynamic') of women conceiving by ART with absent corpus luteum and circulating relaxin, particularly during early pregnancy, consistent with the rat studies (Conrad and Baker, 2013). In support of this concept is a small pilot study conducted throughout the first trimester of women with ovarian failure who conceived using donor oocytes; in these women there was a subdued gestational increase in 24-h endogenous creatinine clearance (an estimation of glomerular filtration rate or GFR) and an attenuated decline in plasma osmolality (Smith *et al.*, 2006b). On the other hand, we expect that, relative to spontaneous conceptions, women conceiving through ART with multiple corpora lutea and elevated circulating relaxin will have an exaggerated or 'hyperdynamic' circulation (Conrad and Baker, 2013).

After 8–10 weeks of gestation when the placenta becomes sufficiently mature and functional, we may find that vasodilators of placental origin are sufficient to rescue maternal vasodilation in the gravid women without a corpus luteum (Conrad and Baker, 2013). Nevertheless, there is evidence to suggest that women who develop

early-onset preeclampsia with fetal growth restriction (<34 gestational weeks) or normotensive fetal growth restriction manifest a hypodynamic circulation during early pregnancy with relatively reduced cardiac output and increased systemic vascular resistance relative to women who experience a normal pregnancy (Vasapollo *et al.*, 2004; Salas *et al.*, 2006; De Paco *et al.*, 2008; Khaw *et al.*, 2008). Thus, if women conceiving through ART who lack a corpus luteum and circulating relaxin reveal a hypodynamic circulation during early pregnancy as predicted, then by analogy, they could be at increased risk of developing fetal growth restriction with or without early-onset preeclampsia. In contrast, women who develop late-onset preeclampsia or gestational hypertension may manifest a hyperdynamic circulation during early pregnancy with relatively increased cardiac output and reduced systemic vascular resistance compared to women who experience a normal pregnancy (Easterling *et al.*, 1990; Bosio *et al.*, 1999; De Paco *et al.*, 2008; Khaw *et al.*, 2008). If women conceiving by ART who have multiple corpora lutea and elevated circulating relaxin present with a hyperdynamic circulation during early pregnancy as hypothesized, then again by analogy, they could be at increased risk of developing late-onset preeclampsia or gestational hypertension. Notably, emerging evidence suggests that ART is indeed associated with increased risk for preeclampsia and delivery of small for gestational age infants (Conrad and Baker, 2013). However, it is unknown whether the postulated hypodynamic or hyperdynamic circulation in early pregnancy might contribute to the eventual development of adverse obstetrical outcomes later in gestation reflect a more fundamental pathology or both, and in the case of ART, other factors such as the preexisting infertility or the added immunological challenge of donor oocytes are likely to also play a role (Salha *et al.*, 1999; Jaques *et al.*, 2010).

Relaxin administration mimics the circulatory changes of pregnancy

As summarized in Tables II and III, relaxin administration to rats and humans reproduced many of the circulatory changes observed in normal pregnancy. Essentially, relaxin is a vasodilator in both the renal and systemic circulation of conscious rats and humans (Danielson *et al.*, 1999; Danielson and Conrad, 2003; Conrad *et al.*, 2004; Bogzil *et al.*, 2005; Smith, *et al.*, 2006a; Dschietzig *et al.*, 2009; Khanna *et al.*, 2009; Conrad, 2011; Ponikowski *et al.*, 2014; Voors *et al.*, 2014), although the influence of relaxin on cardiac output in humans is controversial, but has only been tested in the setting of heart failure and not in normal human volunteers (Dschietzig *et al.*, 2009; Ponikowski *et al.*, 2014). Relaxin infusion in conscious rats rapidly increased renal blood flow and glomerular filtration rate, which was sustained upon long-term administration (Danielson *et al.*, 1999; Danielson and Conrad, 2003). Comparable findings were observed in women, with the exception that increased glomerular filtration was observed only after a longer duration of administration (Smith *et al.*, 2006a; Dschietzig *et al.*, 2009; Khanna *et al.*, 2009; Weiss *et al.*, 2009; Conrad and Davison, 2014). Finally, relaxin administration to conscious rats blunted the systemic and renal pressor responses to angiotensin II (Danielson *et al.*, 1999; Debrah *et al.*, 2005), and increased global arterial compliance (Conrad *et al.*, 2004).

Early morphological findings of hepatic sinusoids obtained from rats administered relaxin were indicative of reduced hepatic vascular resistance and increased hepatic blood flow consistent with the mobilization of splanchnic blood to augment venous return, as observed in normal human pregnancy (Bani *et al.*, 2001). More recently, functional evidence supports this concept, revealing that relaxin reduced portal venous pressure and increased portal venous blood flow in a rat model of portal hypertension through two mechanisms: augmentation of NO production by hepatic sinusoidal endothelial cells that, in turn, inhibited hepatic stellate cell contraction, and downregulation of hepatic stellate cell contractile filament expression (Fallowfield *et al.*, 2014).

Small renal arteries isolated from rats and mice administered relaxin were more compliant and demonstrated inhibition of myogenic constriction *ex vivo* (Novak *et al.*, 2002; Conrad *et al.*, 2004; Debrah *et al.*, 2011; McGuane *et al.*, 2011a). Incubation of small renal arteries from rats and mice, as well as subcutaneous arteries from humans, with the hormone also inhibited myogenic constriction (McGuane *et al.*, 2011a). Relaxin elicited a rapid relaxation response in pre-constricted small renal arteries from rats and mice, as well as gluteal and subcutaneous arteries, but not pulmonary resistance arteries from humans (Fisher *et al.*, 2002; McGuane *et al.*, 2011b), and in hamster, cremaster muscle arterioles *in situ* (Willcox *et al.*, 2013). Moreover, relaxin potentiated endothelium-dependent vasorelaxation by bradykinin in mesenteric arteries isolated from male rats treated with the hormone *in vivo* (Leo *et al.*, 2014). Relaxin was shown to increase uterine blood flow velocity in conscious, non-pregnant rats and augment uterine artery compliance during pregnancy in rats (Vodstrcil *et al.*, 2012). Finally, when administered to mice, relaxin increased circulating bone marrow-derived angiogenic progenitor cells and their incorporation into the vasculature of subcutaneous matrigel plug implants (Segal *et al.*, 2012). The hormone also increased nitric oxide production by isolated human $CD34^+$ cells and stimulated their migration (Segal *et al.*, 2012).

Rationale for the potential therapeutic use of relaxin in preeclampsia: exploiting the beneficial hemodynamic attributes of relaxin

During the overt, clinical phase of disease, circulating concentrations of immunoreactive relaxin in preeclampsia appear to be comparable to normal pregnancy (Szlachter *et al.*, 1982). However, recent evidence suggests that women with serum levels in the lowest 10th centile during the first trimester are at increased risk of developing late, but not early-onset preeclampsia (Jeyabalan *et al.*, 2009; Post Uiterweer *et al.*, 2014). Whether the bioactivity of circulating relaxin or RXFP1 signaling is impaired in preeclampsia has not been evaluated. Nor is it known whether relaxin locally produced by tissues such as arteries, placenta and decidua may be reduced. (The reader is referred to several excellent reviews on local relaxin-ligand expression in uterine and other tissues: Kong *et al.*, 2010; Bathgate *et al.*, 2013; Anand-Ivell and Ivell, 2014). Nevertheless, whether or not circulating or locally produced relaxin is deficient, thereby potentially contributing to disease pathogenesis, administration of the hormone to reach supra-physiological concentrations may prove salutary as a therapeutic strategy. This may be particularly apropos for pregnant

Table II Relaxin administered to conscious, non-pregnant rats and humans recapitulates many aspects of the maternal systemic circulatory adaptations of pregnancy.

Circulatory adaptation	Pregnancy		Relaxin	
	Rat	Human	Rat	Human
Cardiac output	↑	↑	↑	+/−[a]
Systemic vascular resistance	↓	↓	↓	↓
Portal venous blood flow	?	↑	↑	?
Arterial compliance	↑	↑	↑	?
Angiotensin II pressor response	↓	↓	↓	?
Myogenic constriction	↓	↓	↓	↓
Circulating bone marrow-derived angiogenic progenitor cell number and/or activity	↑ (mice)[b]	↑	↑ (mice)	?

Relaxin administered to non-pregnant females and males. ?, not tested, ↑ increased, ↓ decreased: pregnancy relative to the non-pregnant state or relaxin administration in non-pregnant rats and humans relative to vehicle infusion. See text for further details and references.
[a]The influence of relaxin on cardiac output in humans is disputed, but has only been tested in the setting of heart failure and not in normal human volunteers (see text for citations).
[b]Segal, Sautina, Li and Conrad, unpublished.

Table III Relaxin administered to conscious, non-pregnant rats and humans recapitulates many aspects of the maternal renal circulatory and osmoregulatory changes of pregnancy.

Circulatory or osmoregulatory adaptation	Pregnancy		Relaxin	
	Rat	Human	Rat	Human
Glomerular filtration rate	↑	↑	↑	+/− ↑[a]
Renal plasma flow	↑	↑	↑	↑
Renal vascular resistance	↓	↓	↓	↓
Angiotensin II-induced renal vasoconstriction	↓	?	↓	?
Plasma osmolality	↓	↓	↓	?

Relaxin administered to non-pregnant females and males. ?, not tested, ↑ increased, ↓ decreased: pregnancy relative to the non-pregnant state or relaxin administration in non-pregnant rats and humans relative to vehicle infusion. See text for further details and references.
[a]Chronic, not acute relaxin administration.

women conceiving by ART who lack a corpus luteum, and hence, any circulating relaxin, and who are at increased risk of developing preeclampsia.

As described above, in many respects, the circulatory pattern in preeclampsia at least during the overt phase of the disease is the antithesis of normal pregnancy. Because relaxin administration can reproduce many of the circulatory changes of normal pregnancy, and indeed, mediates these changes in gravid rats and possibly in pregnant women, too, it is logical to investigate whether relaxin administration to preeclamptic women could restore, or at least partially so, circulatory changes to normal pregnancy levels (Table IV). As a consequence of its pleiotropic hemodynamic properties outlined above, relaxin treatment would be expected to relieve one of the fundamental pathologies of preeclampsia, maternal organ vasoconstriction. Presuming that myocardial function is not unduly compromised, relaxin would be anticipated to improve cardiac output due to a concurrent reduction in systemic vascular resistance and augmentation of venous return, as well as ameliorate systemic and renal vasoconstriction, thereby improving maternal organ perfusion. A reciprocal rise in cardiac output should buffer the decline in arterial pressure as a consequence of the beneficial reduction in systemic vascular resistance, thereby preserving uterine blood flow, which may not have an efficient autoregulatory capacity. If unacceptably high arterial pressure persists despite relaxin treatment, routine anti-hypertensive therapy could be implemented. Finally, relaxin may be further beneficial by increasing uterine blood flow in preeclampsia (Vodstrcil *et al.*, 2012; Gooi *et al.*, 2013).

Relaxin administration would be expected to reverse robust myogenic constriction (Debrah, Novak and Conrad, unpublished preliminary data; Fig. 1), as well as blunt systemic and renal pressor responses to activation of the angiotensin II receptor, type 1 (AGTR1) by circulating and locally derived angiotensin II, and AGTR1 autoantibodies (Wallukat *et al.*, 1999), thereby reducing arterial tone. In the same vein, relaxin should blunt the response to endogenous vasoconstrictors in uterine spiral arteries with failed remodeling and retained smooth muscle, thereby improving uteroplacental blood flow. In addition to possible improvements of vessel wall geometry and composition given a sufficient duration of relaxin infusion, vasodilation or reduction in arterial tone would be anticipated to improve arterial compliance in preeclampsia (Conrad and Shroff, 2011).

Table IV Expected improvements of cardiovascular and renal function in preeclampsia during recombinant human relaxin (rhRLX) administration.

Preeclampsia	Preeclampsia + rhRLX
Systemic and renal vasoconstriction	Systemic and renal vasodilation
↑ Sensitivity to vasoconstrictors	↓ Sensitivity to vasoconstrictors
↓ Arterial compliance	↑ Arterial compliance
↑ Myogenic constriction	↓ Myogenic constriction
↓ Uterine blood flow	↑ Uterine blood flow
↓ Circulating bone marrow-derived angiogenic progenitor cells and activity	↑ Circulating bone marrow-derived angiogenic progenitor cells and activity
Endothelial dysfunction	Improved endothelial function
Vascular inflammation	Reduced vascular inflammation
Insulin resistance	Improved insulin resistance
Cytotrophoblast apoptosis and necrosis	Reduced cytotrophoblast apoptosis and necrosis

↑ increased, ↓ decreased: preeclampsia relative to normal pregnancy; preeclampsia + rhRLX vs preeclampsia. See text for further details and references.

Through promoting skeletal muscle vasodilation, thus enhancing insulin-mediated glucose disposal, relaxin should ameliorate insulin resistance in preeclampsia (Bonner *et al.*, 2013). Finally, by mobilization and activation of bone marrow-derived angiogenic progenitor cells, the hormone would be expected to repair injured endothelium and improve maternal vascularity in organs such as breast, uterus, pancreas, skin and fat. Formation of new or remodeling of existing blood vessels by relaxin would also contribute to the decline in systemic vascular resistance and increase in global arterial compliance. Table IV summarizes some of the theoretical rationale based on relaxin's salutary physiological attributes for underwriting preclinical and clinical investigation of the hormone as a potential therapeutic in preeclampsia, by granting agencies and pharmaceutical companies.

Rationale for the potential therapeutic use of relaxin in preeclampsia: exploiting the beneficial molecular attributes of relaxin

As a consequence of its pleiotropic molecular properties, relaxin treatment would be expected to improve many aspects of maternal circulatory pathology in preeclampsia. Of course, these pleiotropic molecular properties ultimately underlie the beneficial hemodynamic actions of relaxin as discussed above. Many of the vasodilatory effects of relaxin appear to be mediated through endothelium-derived NO (Conrad, 2010). The rapid vasodilatory response (within minute(s)) observed in small renal arteries isolated from rats and mice, as well as human subcutaneous arteries is mediated through Gαi/o protein coupling to PI3 kinase, protein kinase B, and endothelial NO synthase, but not VEGF receptor transactivation or increased endothelial cell calcium (McGuane *et al.*, 2011b; Sarwar *et al.*, 2015). The sustained vasodilatory response (hour(s) to day(s)) involves upregulation of arterial gelatinase activities by relaxin that, in turn, hydrolyze big ET (endothelin) to ET_{1-32}, thereby activating the endothelial ET_B receptor-NO vasodilatory pathway (Jeyabalan *et al.*, 2003). Though a logical extension of these findings, whether relaxin also upregulates the endothelial ET_B receptor is disputed (Dschietzig *et al.*, 2003; Kerchner *et al.*, 2005; Sarwar *et al.*, 2015). By increasing endothelial NO production, relaxin could contribute to therapeutic vasodilation in preeclampsia. Interestingly, the sustained vasodilatory pathway critically depends on arterial-derived VEGF and PLGF, but the underlying mechanisms are presently unknown (McGuane *et al.*, 2011a). One possibility is that relaxin increases arterial VEGF and PLGF activities, and thus, could mitigate the detrimental effects of circulating sVEGF-R1 on endothelial cell health in preeclampsia by improving local, arterial angiogenic balance and nitric oxide production. Another interesting, but untested possibility is that RXFP1 may heterodimerize with the AGTR2 receptor in the endothelium (by analogy to myofibroblasts (Chow *et al.*, 2014)), thereby promoting NO production and vasodilation. Indeed, pharmacological blockade or genetic deletion of the AGTR2 receptor prevented the physiological decline of arterial pressure during early gestation in mice (Chen *et al.*, 2007; Carey and Rose, 2011).

Recent evidence has suggested that relaxin abrogates angiotensin II-induced increases of intracellular calcium in cultured rat mesangial cells independently of NO or AGTR2 (Carvalho *et al.*, 2012). Reductions in both basal and angiotensin II-induced cytosolic calcium by relaxin have also been observed in human mesangial cells (Costers and Conrad, unpublished). Theoretically, this action might contribute to the gestational increases of glomerular filtration in normal pregnancy by relaxing mesangial cells and increasing surface area for filtration. If these findings in mesangial cells carry over to vascular smooth muscle, then vasodilation would result. Interestingly, relaxin elicits comparable systemic vasodilation in normotensive control rats with normal endothelial function and in rats with genetic hypertension (spontaneously hypertensive rats) or angiotensin II-induced hypertension with some degree of endothelial dysfunction (Debrah *et al.*, 2005). Thus, despite endothelial dysfunction, relaxin would be expected to exert a vasodilatory response in preeclampsia by directly acting on vascular smooth muscle to lower intracellular calcium.

Relaxin was recently shown to confer anti-inflammatory properties, which may ultimately prove therapeutic in preeclampsia (Dschietzig *et al.*, 2012; Brecht *et al.*). Specifically, relaxin mitigated TNF-α-induced increases of vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) expression in cultured human umbilical vein

endothelial cells (HUVEC). Relaxin also reduced a TNF-α-mediated increase in chemokine receptor type-1 (CCR-1) secretion by THP-1 cells, a human monocyte leukemia cell line, and suppressed THP-1 adhesion to a monolayer of HUVEC (Brecht *et al.*). Moreover, the hormone improved acetylcholine-induced relaxation in rat aortic rings that were incubated with TNF-α (Dschietzig *et al.*, 2012). The mechanisms underlying this beneficial action were several-fold: enhanced eNOS activity, but not expression through increased phosphorylation of Ser1177 and Ser633 and reduced phosphorylation of Thr495, inhibited arginase II expression and increased superoxide dismutase-1 (SOD-1) that, in turn, mitigated oxidative and nitrosative stress (Dschietzig *et al.*, 2012). Because circulating TNF-α and adhesion molecules are increased in preeclampsia and endothelium-dependent relaxation is impaired (Conrad and Benyo, 1997), relaxin administration may be beneficial in preeclampsia by reducing vascular inflammation. Moreover, in the setting of chronic angiotensin II-induced hypertension, relaxin decreased arterial pressure coincident with reductions in renal oxidative stress and circulating dimethylarginine that resulted in increased NO bioavailability (Sasser *et al.*, 2014). Because a prominent pathological feature of preeclampsia is enhanced vascular AGTR1 activity, oxidative stress and circulating ADMA (Fickling *et al.*, 1993; Hubel, 1999), relaxin administration would be anticipated to mitigate this pathology. Thus, there is good reason to consider relaxin administration in the context of improving endothelial health and ameliorating vascular inflammation.

Interestingly, relaxin was reported to activate PPAR-γ transcriptional activity in a ligand-independent fashion through increasing expression of PPAR-γ coactivator 1α (PCG1α) (Singh *et al.*, 2015). Deficiency of placental PPAR-γ activity has been implicated in the pathogenesis of preeclampsia (McCarthy *et al.*, 2013). Antagonism of PPAR-γ in pregnant rats produced a syndrome resembling preeclampsia (McCarthy *et al.*, 2011a) and PPAR-γ activation by rosiglitazone in a rat model of preeclampsia ameliorated disease symptoms (McCarthy *et al.*, 2011b). Thus, relaxin may also be therapeutic in preeclampsia from the standpoint of enhancing placental PCG1α expression, and hence, PPAR-γ transcriptional activity.

Rationale for the potential therapeutic use of relaxin in preeclampsia: exploiting the cell survival properties of relaxin

Placental cells, cytotrophoblast (CTB), undergo accelerated apoptosis and cell death in preeclampsia (Al-Nasiry *et al.*, 2006; Heazell and Crocker, 2008). Relaxin administration would be expected to exert cytoprotective action in CTB (and maternal endothelium) because the hormone activates the well-known cell survival pathway, PI3 kinase/Akt (Luo *et al.*, 2003; McGuane *et al.*, 2011b; Cudmore *et al.*, 2012). Moreover, relaxin is likely to increase NO production in CTB, which is also cytoprotective (Dash *et al.*, 2003). Relaxin was shown to be anti-apoptotic in epithelial and stromal cells of the mouse vagina and cervix, as well as in prostate cancer cells and rat neonatal cardiomyocytes (Feng *et al.*, 2007; Moore *et al.*, 2007; Yao *et al.*, 2008), and more recently in the CTB cell line, HTR-8/SVneo (Campo *et al.*, 2013; Lodhi *et al.*, 2013). Importantly, both adult endothelial and CTB cells express the major relaxin receptor, RXFP1 ((Maruo *et al.*, 2007), Conrad, unpublished). In addition to mitigating apoptosis, relaxin may also promote CTB invasion (Maruo *et al.*, 2007; Post Uiterweer *et al.*, 2012), which is impaired in preeclampsia resulting in deficient remodeling of uterine spiral arteries, and consequently, compromise of blood flow to the placenta.

Rationale for the potential prophylactic use of relaxin in preeclampsia

The origin of preeclampsia is widely believed to involve defective trophoblast invasion, and hence, impaired placentation. Because these deficits transpire in early pregnancy, molecular pathology of delivered placentas may not be informative. Recent DNA microarray analyses and bioinformatics identified differentially expressed genes between early placentas (chorionic villous samples; ~11.5 gestational weeks) from women who developed preeclampsia 5–6 months later and those who experienced an uncomplicated pregnancy (Founds *et al.*, 2009; Rabaglino *et al.*, 2015). These differentially expressed genes were evaluated in the context of DNA microarray databases in the public domain that were derived from endometrial biopsies obtained during the menstrual cycle and early pregnancy in healthy subjects. The bioinformatics revealed provocative, but compelling evidence for insufficient or defective endometrial maturation (decidualization) including reduced uterine natural killer cell number and/or activity in the late secretory phase and during early pregnancy in the women who developed preeclampsia (Rabaglino *et al.*, 2015). This work provided the heretofore missing, but essential prospective data supporting the hypothesis that the genesis of impaired trophoblast invasion and placentation in preeclampsia could reside in a poorly developed endometrium.

Interestingly, expression of endometrial relaxin increases upon decidualization, and relaxin itself induces decidualization. The combination of methoxyprogesterone acetate and relaxin produced a synergistic increase in prolactin and insulin growth factor binding protein-1 (IGFBP-1) secretion from human endometrial stromal cells, two classic biomarkers of decidualization (Lane *et al.*, 1994). Moreover, relaxin increased transcription of prolactin and IGFBP-1 in human decidual and endometrial stromal cells (Tang *et al.*, 2005). The hormone also stimulated VEGF secretion in cultured human endometrial cells (Unemori *et al.*, 1999), and in cultured glandular epithelial and stromal cells isolated from secretory phase endometrium (Palejwala *et al.*, 2002). Consistent with an *in vivo* study described below, relaxin stimulated glycodelin mRNA and protein in cultured human glandular epithelial cells, another biomarker of decidualization (Tseng *et al.*, 1999). Relaxin synergized with phosphodiesterase inhibition to promote decidualization of human endometrial stromal cells as reflected by increased prolactin and IGFBP-1 secretion (Bartsch *et al.*, 2004). The hormone was also reported to increase its own receptor, RXFP1, and IGFBP-1 in human endometrial-decidual cells (Mazella *et al.*, 2004). Finally, relaxin was shown to induce decidualization of cultured endometrial stromal cells, mediated through IL-11 production and action (Dimitriadis *et al.*, 2005).

Progesterone-dependent expression of relaxin protein was detected by immunohistochemistry in human secretory endometrium (Yki-Jarvinen *et al.*, 1985). Again using immunohistochemistry, relaxin expression was marked in glandular epithelium, endometrial stromal

cells and decidua beginning in the mid-secretory endometrium and extending through pregnancy (Bryant-Greenwood *et al.*, 1993). Serum concentrations of relaxin and glycodelin secreted by the corpus luteum and endometrium, respectively, coincided during the late luteal phase and early pregnancy suggesting that relaxin stimulates glycodelin secretion (Stewart *et al.*, 1997). The relaxin receptor, RXFP1, increased expression and ligand binding activity starting with the early secretory phase in the human endometrium (Bond *et al.*, 2004). Finally, when administered to ovariectomized, steroid replaced rhesus monkeys, relaxin increased uterine weight and angiogenesis, as well as lymphocyte number, all hallmarks of decidualization (Goldsmith *et al.*, 2004).

If the antecedents of preeclampsia reside in poorly decidualized endometrium and relaxin has the potential to promote decidualization, then conceivably relaxin could be administered in the late secretory phase and during early pregnancy to improve endometrial maturation, thereby reducing the risk of developing preeclampsia. Thus, women with a history of severe preeclampsia or who have comorbidities that increase the risk of preeclampsia such as obesity, chronic hypertension, diabetes, autoimmune disease or polycystic ovary syndrome might be treated empirically with supplemental relaxin administration to improve endometrial maturation. Indeed, these comorbidities themselves may impair endometrial maturation in the secretory phase (Schulte *et al.*, 2015). Alternatively, women who manifest impaired endometrial maturation might be identified by an abnormal molecular signature on a diagnostic endometrial biopsy or in uterine secretions during the late secretory phase or by circulating biomarkers indicative of inadequate decidualization. The idea that relaxin might be administered in the late secretory phase and early pregnancy to improve endometrial function (and possibly maternal circulatory adaptations as well), in order to reduce the risk of preeclampsia and perhaps of other placental syndromes is a new concept that requires future testing.

Relaxin safety profile

Based on its reported matrix-degrading attributes and a favorable Phase II trial, a larger randomized, double-blinded, placebo-controlled trial was undertaken to investigate subcutaneous administration of recombinant human relaxin for 24 weeks in patients with stable and diffuse, moderate to severe scleroderma (Khanna *et al.*, 2009). Primary and secondary outcomes related to improvement in disease pathology were not met. Of notable concern was an overall decline of ~10 ml/min from baseline in predicted creatinine clearance after abrupt cessation of therapy, which was not observed in the placebo-controlled cohort. There were also serious adverse renal events consisting of doubling of serum creatinine, renal crisis or severe rebound hypertension in 6 of 169 subjects after abrupt cessation of relaxin. Thus, treatment was tapered in the remaining 62 patients of this study with only 1 serious adverse event. It is possible that these serious adverse events were related to the long-term duration of the relaxin exposure or to the underlying pathology (scleroderma). Nevertheless, the experience strongly suggested that cessation of relaxin therapy should be gradual especially after prolonged administration, and arterial pressure and renal function should be frequently monitored during tapering.

There is extensive experience with short-term intravenous infusion (<48 h) of recombinant human relaxin in heart failure (Dschietzig *et al.*, 2009; Teerlink *et al.*, 2009; Teerlink *et al.*, 2013). Although hypotension was more frequent in relaxin cohorts, this complication was mitigated by selecting patients with systolic blood pressure >125 mmHg and by closely monitoring blood pressure and adjusting the infusion rate or stopping the infusion as needed using prespecified guidelines. Otherwise, adverse events were comparable between the relaxin and placebo groups, and relaxin appeared to be well tolerated and safe. Intriguingly, survival at 180 days was significantly improved in the relaxin-treated group.

In a double-blind, placebo-controlled trial, recombinant human relaxin was infused for 24-h in women >40 gestational weeks scheduled for labor induction (Weiss *et al.*, 2009). Contrary to the investigators' hypothesis, relaxin did not accelerate cervical ripening, reduce the time to delivery or elevate the incidence of spontaneous labor or the onset of active labor, relative to placebo. However, consistent with the cardiorenal actions of relaxin as described above, the hormone decreased serum creatinine and blood urea nitrogen, increased predicted creatinine clearance, and produced a small decrease in systolic blood pressure. No adverse side effects were reported.

Theoretically, a potentially deleterious consequence of relaxin administration could arise in the setting of pathological oxidative stress as in preeclampsia, where more NO produced in response to the hormone could react with superoxide to form the potentially damaging free radical, peroxynitrite (Pacher *et al.*, 2007). However, several investigators have found that relaxin reduces oxidative stress in the setting of pathological challenges, albeit outside of preeclampsia (Dschietzig *et al.*, 2012; Sasser *et al.*, 2014). To date, relaxin has been administered as a recombinant protein, and as such, it has the theoretical potential to elicit antibody formation some of which could be neutralizing. Like oestrogen and other growth factors, relaxin may promote some tumors such as prostate and breast (Feng *et al.*, 2007; Binder *et al.*, 2014). The relatively short time course of administration that we envision for preeclampsia therapy would seem to present minimal risk in this regard, although preeclamptic woman with known cancer or at high risk would clearly be excluded. Finally, the consequences of fetal exposure are unknown, although cord blood relaxin is normally a fraction of maternal circulating concentrations, and in rhesus monkeys, there was little transfer from the maternal to the fetal circulation (Weiss *et al.*, 1978; Lucas *et al.*, 1989; Cossum *et al.*, 1991; Johnson *et al.*, 1992). Nevertheless, relaxin cord blood levels at delivery and neonatal/pediatric monitoring would be important endpoints of a clinical trial.

To summarize, relaxin is a naturally occurring molecule that circulates in the luteal phase and during pregnancy, and is expressed locally by many tissues including blood vessels (Novak *et al.*, 2006). Based on the limited number of clinical trials described above, the safety profile of relaxin has so far been favorable particularly when administered short-term, and thus, does not contraindicate its use as a potential therapeutic in preeclampsia. Blood pressure and renal function should be frequently monitored during and after the infusion, which should be tapered and not abruptly stopped. Importantly, relaxin is not expected to accelerate cervical ripening or induce labor. Thus, short-term relaxin administration to women with severe preeclampsia, in order to achieve supra-physiological circulating levels of ≤10 ng/ml would be expected to ameliorate the maternal disease

sufficiently to permit safe prolongation of pregnancy allowing for further fetal maturation.

Conclusions

One approach to drug development is designing pharmaceuticals targeting individual inciting factors. A potential downside is that elimination of only one of several inciting factors that circulate in preeclampsia might be insufficient for some women. For example, in preeclampsia specifically targeting sVEGF-R1 may be inadequate due to other, unopposed pathologic factors, for example, soluble endoglin or AGTR1 activation by autoantibodies. Another potential drawback is that any given pharmaceutical may have untoward side effects, for example, administration of VEGF (previously identified as vascular permeability factor) to neutralize sVEGF-R1 may result in excessive capillary leakage, and treatment with PLGF may exacerbate activation of leukocytes, which are already activated in preeclampsia (Clauss *et al.,* 1996). Likewise, administration of an antibody to bind sVEGF-R1 is likely to antagonize the physiological cellular actions of membrane VEGF-R1, a potentially undesirable consequence.

We propose a nontraditional, but logical approach to developing therapeutics for preeclampsia, that is, the administration of pregnancy hormone(s) that underpin the circulatory adaptations of uncomplicated pregnancy, in order to at least partly restore the maternal circulation to normal in preeclampsia. Mainly based on preclinical (animal) studies, we identified circulating relaxin as a potent vasodilator in the renal and systemic circulations during normal pregnancy. Relaxin exerts pleiotropic molecular and physiological actions, through the GPCR, RXFP1, activating many cell signaling pathways, several of which are likely to be beneficial to women suffering from preeclampsia. Thus, rather than targeting one pathogenic factor, our approach would enable the activation of several potentially salutary signaling pathways and physiological actions simultaneously through the administration of one therapeutic. In other words, given the complex pathogenesis of preeclampsia most likely involving more than one inciting factor and multiple organ dysfunction, a therapeutic with the potential to combat several of these at once would seem to be advantageous. Moreover, relaxin is a naturally occurring protein, which normally circulates in the luteal phase and during pregnancy, and as such, modest elevation of circulating levels by supplementation for days to weeks(s), in order to quell disease pathogenesis and safely prolong gestation is less likely than exogenous compounds to have untoward side effects. As detailed above, relaxin has an encouraging safety profile both outside of and within pregnancy. In particular, when administered to gravid women near term in a clinical trial of cervical ripening, the only effects noted were small decreases in arterial pressure and serum creatinine, both consistent with its known physiological actions. Importantly, the hormone did not promote cervical ripening (Weiss *et al.,* 2009). Again, based on its pleiotropic actions, relaxin may see clinical utility soon in the setting of acute heart failure, another complex disease with multiple pathogenic mechanisms (Teerlink *et al.,* 2013).

For preeclampsia, there would seem to be reasonable justification for evaluating preventative, therapeutic and curative strategies that are plausible, having emerged from sound basic research. In this regard, the strategy proposed herein, we believe, offers a plausible therapeutic approach to preeclampsia that warrants testing. Like insulin, relaxin is currently cumbersome to administer, but there are several laboratories pursuing the development of small molecule, orally bioavailable, RXFP1 mimetics, and these are likely to be a reality soon (e.g. Xiao *et al.,* 2013). Although we hope that statins prove to be effective in treating preeclampsia or as a prophylaxis (Costantine and Cleary, 2013), clinical trials are just beginning and we may not know for some time whether they will be successful or have unforeseen side effects precluding or limiting their use in pregnancy. Even if trials are successful, as pertains to the non-pregnant population, statins may not be tolerated by all women (Matteucci and Giampietro, 2013). Thus, it would seem prudent to have more therapeutic candidates for preeclampsia including relaxin in the pipeline beginning with their preclinical testing, and to have more than one therapeutic option, if at all possible, for the disease.

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Author's role

The author conducted the literature searches, drafted the manuscript, responded to requests for revisions and prepared the final version.

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Conflict of Interest

The author is an inventor or co-inventor of use patents for relaxin, and has served as a paid or unpaid consultant to Connetics, BAS Medical, Corthera, Novartis and other companies concerning the use of relaxin.

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