Relaxin deficiency attenuates pregnancy-induced adaptation of the mesenteric artery to angiotensin II in mice

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Marshall SA, Leo CH, Senadheera SN, Girling JE, Tare M, Parry LJ. Relaxin deficiency attenuates pregnancy‐induced adaptation of the mesenteric artery to angiotensin II in mice. Am J Physiol Regul Integr Comp Physiol 310: R847–R857, 2016.—Pregnancy is associated with reduced peripheral vascular resistance, underpinned by changes in endothelial and smooth muscle function. Failure of the maternal vasculature to adapt correctly leads to serious pregnancy complications, such as preeclampsia. The peptide hormone relaxin regulates the maternal renal vasculature during pregnancy; however, little is known about its effects in other vascular beds. This study tested the hypothesis that functional adaptation of the mesenteric and uterine arteries during pregnancy will be compromised in relaxin-deficient (Rln−/−) mice. Smooth muscle and endothelial reactivity were assessed in small mesenteric and uterine arteries of nonpregnant (estrus) and late-pregnant (day 17.5) wild-type (Rln+/+) and Rln−/− mice using wire myography. Pregnancy per se was associated with significant reductions in contraction to phenylephrine, endothelin-1, and ANG II in small mesenteric arteries, while sensitivity to endothelin-1 was reduced in uterine arteries of Rln+/+ mice. The normal pregnancy-associated attenuation of ANG II-mediated vasoconstriction in mesenteric arteries did not occur in Rln−/− mice. This adaptive failure was endothelium-independent and did not result from altered expression of ANG II receptors or regulator of G protein signaling 5 (Rgs5) or increases in reactive oxygen species generation. Inhibition of nitric oxide synthase with L‐NAME enhanced ANG II‐mediated contraction in mesenteric arteries of both genotypes, whereas blockade of prostanoiid production with indomethacin only increased ANG II‐induced contraction in arteries of pregnant Rln+/+ mice. In conclusion, relaxin deficiency prevents the normal pregnancy-induced attenuation of ANG II‐mediated vasoconstriction in small mesenteric arteries. This is associated with reduced smooth muscle‐derived vasodilator prostanoids.

angiotensin; pregnancy; relaxin; vascular

EARLY MATERNAL VASCULAR ADAPTATIONS to pregnancy are paramount to the survival and development of the fetus and for maternal health. Cardiac output and blood volume increase by 40–50%, whereas peripheral vascular resistance decreases. Global arterial compliance increases in parallel with cardiac output, thereby preserving diastolic pressure (8). These physiological adaptations are necessary to allow the large increase in blood flow to the feto-placental unit, which is required for a healthy pregnancy. Pregnancy is associated with attenuated responsiveness of the systemic vasculature to vasoconstrictors, including the α1‐adrenoceptor agonist phenylephrine (PE) (11), the thromboxane mimetic U46619 (24), and ANG II (17, 39). There is also upregulation of endothelium‐dependent vasodilation (9, 24), involving nitric oxide (NO), prostacyclin (PGI2), and endothelium‐derived hyperpolarization (22, 28, 30). Failure of the maternal systemic vasculature to adapt sufficiently can lead to serious complications, such as pregnancy‐induced hypertension and preeclampsia (3). These conditions are among the leading causes of maternal and fetal morbidity and mortality worldwide.

The peptide hormone relaxin mediates the essential renal hemodynamic adaptations in early pregnancy through direct actions on the renal vasculature (8). In women, serum relaxin concentrations are highest in the first trimester (47) and are correlated with changes in renal artery resistance (1, 40). Women who conceive via egg donation because of ovarian failure (which means they have no measurable circulating relaxin) (23) have attenuated creatinine clearance and increased plasma osmolarity relative to women with normal ovarian function (44). This is consistent with a failure of the renal vasculature to undergo appropriate pregnancy‐related adaptations (7).

Treatment of nonpregnant rats with recombinant human relaxin increases renal plasma flow and glomerular filtration rate, and reduces systemic vascular resistance (10). These beneficial effects of relaxin are underpinned by a reduction in myogenic constriction of small renal arteries (38). Removal of circulating relaxin from midterm pregnant rats using relaxin‐neutralizing antibodies (MCA1) prevents the elevation in renal plasma flow, glomerular filtration rate, cardiac output, and the decrease in systemic vascular resistance observed during normal pregnancy (13, 37). MCA1 treatment during pregnancy also increases small renal artery myogenic reactivity (37) and uterine arterial wall stiffness (50), demonstrating impaired renal artery vasodilation and uterine artery remodeling. Pregnant relaxin gene knockout (Rln−/−) mice have stiffer uterine arteries and give birth to smaller fetuses compared with their wild-type (Rln+/+) counterparts (15). Pregnant women with lower than normal serum concentrations of relaxin are at increased risk of developing late‐onset preeclampsia in combination with a small‐for‐gestational age newborn, evidence linking relaxin with the onset of this pregnancy‐related disease (21, 48). Overall, these studies illustrate the detrimental effects on the vasculature of a lack of circulating endogenous relaxin and the possible consequences during pregnancy.

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Vascular adaptations in the mesenteric arterial bed are important during pregnancy because of the role this vascular bed plays in regulating blood pressure. Studies in nonpregnant rodents support the idea that relaxin acts directly on mesenteric arteries, as relaxin receptors (RXFP1) are localized on the endothelium and vascular smooth muscle cells of these arteries (20). In vivo, relaxin treatment blunts the mesenteric artery response to the vasoconstrictors arginine vasopressin and nor-epinephrine, reduces blood pressure in spontaneously hypertensive rats (31), reduces myogenic reactivity (38), and increases flow-mediated vasodilation (49) through mechanisms involving endothelium-derived NO. Furthermore, bradykinin (BK)-mediated, endothelium-dependent relaxation is enhanced in the mesenteric arteries of relaxin-treated rats, underpinned by increased contributions of NO (20) and prostacyclin (26).

MATERIALS AND METHODS

Animals. All animal experiments were approved by The University of Melbourne Animal Experimental Ethics Committee (AEEC no. 1212387) and were conducted in accordance with the Australian Code of Practice and the National Health and Medical Research Council. This study used wild-type (Rln+/−) and relaxin-deficient (Rln−/−) mice. Homologous recombination in embryonic stem cells was used to disrupt the relaxin gene by deleting a region essential for biological activity and replacing it with the neomycin transgene (51). Rln−/− mice were then backcrossed to a C57BL/6J background, and the F14 generation was relocated to the School of BioSciences. PCR analysis of genomic DNA from ear clips confirmed mouse genotypes, as previously described (15). Mice were maintained on an automated diet (Barastock, VIC, Australia), and water was available ad libitum.

Initially, female adult mice (Rln+/− and Rln−/−) aged 3–5 mo were studied at estrus (virgin, nonpregnant), and on day 17.5 of pregnancy. Once the phenotypes were established, we included one other time point, day 12.5 of pregnancy, to investigate the onset of the phenotype. These time points correspond to absent circulating levels of relaxin (estrus), midrange (12.5), and high (17.5) circulating levels. To select mice in estrus, vaginal smears were obtained, and vaginal epithelial cells and leukocytes were characterized. Female mice were housed with a stud male of matched genotype overnight and then checked for mating plugs the following morning. The presence of a mating plug was an indication of successful mating and was considered day 0.5 of pregnancy.

Isolation of mesenteric and uterine arteries. Mice were euthanized by 2% isoflurane and cervical dislocation. The mesenteric arcade and uterine arteries were isolated and immediately placed in ice-cold Krebs physiological saline solution, PSS; in mmol/l: 120 NaCl, 5 KCl, 1.2 MgSO4, 1.2 K2HPO4, 25 NaHCO3, 11.1 d-glucose, and 2.5 CaCl2, and bubbled with carbogen (95% O2-5% CO2). Small mesenteric arteries (first-order branches of the superior mesenteric artery: outside diameter ~125 μm) and the main uterine artery (outside diameter: estrus ~150 μm; day 17.5 of pregnancy ~210 μm) were isolated, cleared of fat and loose connective tissue, cut into rings 2 mm in length, and mounted on a wire-myograph (model 610M; Danish Myo Technology, Aarhus, Denmark). The remaining arteries were snap frozen in liquid nitrogen and stored at −80°C. After the arteries were mounted on the myograph, they were allowed to stabilize for 15 min before normalization, as described previously (25).

Assessment of vascular reactivity. Vascular reactivity was tested as previously described (20, 27). Briefly, arteries were contracted with high-potassium PSS (KPSS; K+ = 100 mmol/l, isosmotic replacement of Na+ with K+). Subsequently, the integrity of the endothelium was determined by submaximally preconstricting arteries with PE to 50–60% KPSS, and then applying ACh to induce relaxation (10 μmol/l). Arteries with >95% relaxation were deemed suitable for further analysis. In mesenteric arteries, absolute contraction amplitude to KPSS in Rln+/+ did not differ from that of Rln−/− mice, nor did it significantly change from estrus to day 17.5 of pregnancy. Therefore, responses to constrictors are presented as % KPSS. However, absolute contraction amplitude to KPSS did significantly differ in uterine arteries of Rln−/− mice vs. Rln+/+ mice. Consequently, vasoconstrictor responses of uterine arteries are presented as % maximum contraction (% max).

To evaluate smooth muscle reactivity to vasoconstrictors, cumulative concentration-response curves to ANG II (0.1 μmol/l to 0.1 μmol/l), endothelin-1 (ET1; 0.1 nmol/l to 0.1 μmol/l), PE (1 μmol/l to 10 μmol/l), and the thromboxane mimetic U46619 (0.1 nmol/l to 1 μmol/l) were constructed. The overall response to various constrictors over the entire concentration range tested was determined by area under the curve (AUC) analysis of the concentration-response curves (20, 27). To assess endothelial vasodilator function, mesenteric arteries were submaximally precontracted (50% to 70% of KPSS contraction) using PE (0.1 to 3 μmol/l), and cumulative concentration-response curves to the endothelium-dependent agonists ACh (0.1 nmol/l to 10 μmol/l), and bradykinin (BK; 0.1 nmol/l to 1 μmol/l) were determined. Further investigation of the effects of relaxin deficiency on vascular function focused on the ANG II response in mesenteric arteries. To avoid the confounding effects of tachyphylaxis, each artery was exposed to an ANG II dose response curve only once. The influence of the endothelium on vascular smooth muscle reactivity to ANG II was also evaluated in endothelium-denuded arteries. Arteries were considered to be denuded of endothelium if the response to ACh (10 μmol/l) was abolished. In other experiments, responses to ANG II were examined after 20 min of incubation with different combinations of inhibitors, including the N-type calcium channel blocker nitrendipine (R848, 1 μmol/l), the NO synthase inhibitor L-NAME (200 μmol/l), the cyclooxygenase inhibitor indomethacin (Indo; 1 μmol/l), and the combination of both inhibitors. The role of basal NO or vasodilator prostanooids in modulating contraction was determined by subtracting the AUC in L-NAME or Indo from the AUC obtained in the absence of the inhibitors. Similarly, the basal control of both NO and vasodilator prostanooids was determined by subtracting the AUC in L-NAME+Indo from the AUC obtained in the absence of inhibitors. Responses to ANG II were also examined after 20 min of incubation with scavengers of reactive oxygen species (ROS): catalase for H2O2 (1,000 U/ml), 4-hydroxy-TEMPO for extracellular and intracellular O2− (tempol, 10 μmol/l) or the membrane-impermeable O2− scavenger, superoxide dismutase (SOD; 250 U/ml).

RNA extraction and quantitative PCR. Total RNA was isolated from pooled mesenteric artery samples from four mice using TRIzol reagent (Invitrogen, Carlsbad, CA), as previously described with the following modifications (25). RNA pellets were resuspended in 11 μl RNA Secure (Ambion, Scoresby, VIC, Australia). The concentration of RNA was determined using a NanoDrop ND100 Spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia) with A260: A280 absorbance ratios of >1.8, indicating a sufficient quality of RNA for PCR analysis. First-strand cDNA synthesis used 0.7 μg total RNA in a 20-μl reaction, using random hexamers (50 ng/μl), 10 mM dNTP, and 200 U SuperScript III (Invitrogen, Mulgrave, VIC, Australia). First-strand cDNA synthesis for all samples were performed simultaneously at 25°C for 10 min, 50°C for 5 min, and 85°C for 5 min.
Table 1. Reactivity of mesenteric arteries of wild-type (Rln+/+) and relaxin-deficient (Rln−/−) mice at estrus and on day 17.5 (D17.5) of pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Estrus</th>
<th>D17.5</th>
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<tr>
<td><strong>ET1</strong></td>
<td></td>
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<tr>
<td>E_max</td>
<td>112.9 ± 6.2</td>
<td>94.4 ± 3.1*</td>
</tr>
<tr>
<td>pEC50</td>
<td>8.1 ± 0.1</td>
<td>8.1 ± 0.1</td>
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<tr>
<td><strong>U46619</strong></td>
<td></td>
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<tr>
<td>E_max</td>
<td>108.6 ± 9</td>
<td>111.9 ± 10.7</td>
</tr>
<tr>
<td>pEC50</td>
<td>8.2 ± 0.3</td>
<td>8.2 ± 0.1</td>
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<tr>
<td><strong>PE</strong></td>
<td></td>
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<tr>
<td>E_max</td>
<td>119.9 ± 5.8</td>
<td>107.5 ± 6.6</td>
</tr>
<tr>
<td>pEC50</td>
<td>6.6 ± 0.3</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td><strong>ANG II</strong></td>
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<tr>
<td>E_max</td>
<td>80.8 ± 5.7</td>
<td>35.3 ± 6.1*</td>
</tr>
<tr>
<td>pEC50</td>
<td>8.3 ± 0.1</td>
<td>8.4 ± 0.1</td>
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Values are expressed as means ± SE. E_max is calculated as % KPSS. *Significantly different compared with estrus within genotype. #Significantly different compared with Rln+/+ mice at D17.5.

Quantitative RT-PCR (qPCR) was performed to assess the relative expression of ANG II receptor type 1a (Agtr1a), type 2 (Agtr2), and regulator of G protein signaling 5 (Rgs5) in the mesenteric artery of Rln+/+ and Rln−/− mice in estrus and on day 17.5 of pregnancy. Because of the limited tissue availability, the number of genes investigated was restricted. Forward and reverse primers and 6-carboxy fluorescein-labeled TaqMan probes specific for mouse genes were designed to span an intron/exon junction (Biosearch Technologies, Novato, CA). All quantitative PCR reactions for each gene were performed on one plate, in 10-μl 96-well reactions containing SensiFASTProbe Lo-Rox (Bio-Rad, West Ryde, NSW, Australia), using the Viia7 real-time PCR System (Life Technologies, Mulgrave, VIC, Australia). Negative-template controls substituting cDNA with water or reverse transcriptase-negative controls substituting the reverse transcriptase in the cDNA synthesis were included on each plate. The common housekeeping gene Ribosomal 18s (R18s) was the reference gene. Negative-template controls substituting cDNA with water or RT-negative controls substituting the RT in the cDNA synthesis were included on each plate. For each sample, the mean 18s C_T triplicate value was subtracted from the mean gene of interest triplicate C_T value to normalize gene of interest expression to the reference gene. These normalized data (ΔC_T) were then analyzed using the 2^−ΔΔC_T method and presented as means ± SE.

Reagents for vascular reactivity studies. All drugs were purchased from Sigma-Aldrich (St. Louis, MO), except for U46619 (Cayman Chemical, Ann Arbor, MI). They were all dissolved in distilled water, with the exception of indomethacin, which was dissolved in 0.1 mol/l sodium carbonate, and U46619, which was dissolved in 100% ethanol (final concentration less than 0.1% ethanol) as 1 mmol/l stock solution. All subsequent dilutions were in distilled water.

Statistical analyses. All results are expressed as the means ± SE; n represents the number of animals per group. Concentration-response curves were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 5.0, GraphPad Software, San Diego, CA) to calculate the sensitivity of each agonist (pEC50). Maximum contraction (E_max) to ANG II, ET1, PE, and U46619 was measured as a percentage of contraction to KPSS. Maximum relaxation (R_max) to endothelial agonists (ACh and BK) was expressed as a percentage of the level of preconstriction to PE in mesenteric and uterine arteries. In uterine arteries, constriction evoked by ANG II, ET1, PE, and U46619 was expressed as a percentage of the maximum contraction. Group AUC, pEC50, R_max, and E_max values were compared within or between the single variable of genotype using one-way ANOVA with post hoc analysis using Dunnett’s test, or independent t-tests. Two-way ANOVA with Bonferroni post hoc analysis was used to analyze AUC, pEC50, and E_max across genotype and pregnancy. Gene expression data were analyzed using a two-way ANOVA, with Bonferroni post-hoc analysis. P < 0.05 was considered statistically significant.

Results

Mesenteric responsiveness to vasoconstrictors in virgin mice. ET1, U46619, PE, and ANG II-evoked concentration-dependent vasoconstriction of mesenteric arteries from virgin mice in estrus. Sensitivity, maximal response, and AUC of contractions evoked by each vasoconstrictor were not significantly different between mesenteric arteries from Rln+/+ and Rln−/− mice (Figs. 1, A and C, and 2A, Table 1).

Pregnant Rln−/− mice have mesenteric artery dysfunction. To explore whether relaxin deficiency in pregnant female mice altered vascular reactivity, responses to the cumulative addition of vasoconstrictors and vasodilators were assessed. Maximal contraction to ET1 and AUC, but not pEC50, was significantly reduced (Rln+/+: AUC: P = 0.016, E_max: P = 0.024; Rln−/−: AUC: P = 0.001, E_max: P = 0.019) in late pregnancy in arteries from both Rln+/+ and Rln−/− mice relative to estrus (Table 1). However, there were no differences between genotypes (Fig. 1B). Neither relaxin deficiency nor pregnancy had a significant effect on constriction to U46619 (Fig. 1D, Table 1).

Maximal contraction and sensitivity to PE in arteries of pregnant Rln+/+ mice did not differ significantly from virgin mice (Table 1), but the AUC was significantly (P = 0.002) reduced (Fig. 2). A comparison between genotypes revealed a significantly (P = 0.002) greater AUC in arteries of late-pregnant Rln−/− mice, demonstrating greater contraction to PE over the entire concentration range tested (Fig. 2B).

Pregnancy had a profound effect on the contraction evoked by ANG II in mesenteric arteries of Rln+/+ mice relative to estrus (Fig. 3, A and B). Although pEC50 was unaltered, maximal contraction and AUC were both significantly (P < 0.001) reduced on day 17.5 in mesenteric arteries of pregnant Rln+/+ mice compared with those of mice in estrus (Fig. 3, A–C, Table 1). In contrast, there was no pregnancy-related attenuation of ANG II contraction in the mesenteric arteries of pregnant Rln−/− mice on day 17.5 pregnancy, demonstrated by significantly larger AUC (P = 0.005) and E_max (P = 0.008) compared with Rln+/+ mice (Fig. 3C, Table 1). On day 12.5 of
pregnancy, there was a slight decrease in the AUC in arteries of the Rln^+/+ mice, but this was not significant compared with mice in estrus (Fig. 3C). Responses to ET1, U46619, and PE were unchanged between genotypes at day 12.5 of pregnancy (data not shown).

Relaxin deficiency has no effect on endothelium-dependent relaxation in mesenteric arteries. To evaluate whether relaxin deficiency reduces vasodilator function of mesenteric arteries in late pregnancy, responses evoked by two endothelial agonists, ACh and BK, were examined. There were no significant effects of relaxin deficiency on maximal relaxation, pEC50, or AUC for either ACh or BK (data not shown).

Responsiveness of the uterine artery to vasoconstrictors in virgin and late-pregnant mice. To explore whether the vascular phenotypes of the mesenteric artery in Rln^-/- pregnant mice extended to other vascular beds, we examined reactivity of the main uterine artery. ET1, U46619, PE, and ANG II-evoked concentration-dependent vasoconstriction of main uterine arteries from virgin mice in estrus and on day 17.5 of pregnancy in Rln^+/+ and Rln^-/- mice (Fig. 4). Vasoconstrictor sensitivity and AUC were not significantly different between uterine arteries from Rln^+/+ and Rln^-/- mice in estrus (Table 2). Unlike the mesenteric arteries, the vasoconstrictor responsiveness to PE and ANG II on day 17.5 of pregnancy was comparable between uterine arteries from Rln^+/+ and Rln^-/- mice (Fig. 4).

Mechanisms of ANG II-induced vascular dysfunction in mesenteric arteries of Rln^-/- pregnant mice. To further explore the mechanisms underlying enhanced ANG II-mediated contraction in mesenteric arteries of late-pregnant Rln^-/-

![Fig. 1. Relaxin deficiency has no effect on contractions mediated by endothelin 1 (ET1) and U46619 in mesenteric arteries. Concentration-response curves (left) and area under the curve (AUC) (right) for ET1- (A, B; n = 4–7) and U46619-mediated contractions (C, D; n = 4–7) in mesenteric arteries isolated from (●) wild-type (Rln^+/+) and (◇) relaxin-deficient (Rln^-/-) mice at estrus and on day 17.5 (D17.5) of pregnancy.](http://ajpregu.physiology.org/)
mice, we analyzed expression of ANG II receptor type 1a (Agtr1a), ANG II receptor type 2 (Agtr2), and regulator of G protein signaling 5 (Rgs5) in estrus (Fig. 5, A, C, and E) and day 17.5 of pregnancy (Fig. 5, B, D, and F). Rgs5 regulates blood pressure and vascular ANG II responsiveness in pregnancy (18, 19). Expression of these genes did not differ significantly between Rln+/+ and Rln−/− mice on estrus or day 17.5 of pregnancy.

Enhanced contraction to ANG II has been attributed to increased ROS generation in human omental arteries from preeclamptic women (33). We sought to investigate the possibility that the failure of the mesenteric artery in Rln−/− mice to adapt to pregnancy was due to enhanced ROS generation. Reactivity to ANG II was examined in the absence, or after the arteries had been incubated the ROS scavengers catalase, tempol, or SOD. None of these treatments had a significant effect on the contraction to ANG II in either Rln+/+ or Rln−/− mice on day 17.5 of pregnancy (Fig. 6).

We previously demonstrated that mesenteric arteries of male Rln−/− mice have compromised vasodilator mechanisms (25). Therefore, we investigated whether an underlying deficit in basal NO or vasodilator prostanoids contributed to the failure of the mesenteric artery of Rln−/− mice to become less responsive to ANG II in late pregnancy. In a separate series of experiments, ANG II-induced vasoconstriction was evoked in the presence or absence of L-NAME alone, Indo alone, or the...
combination of l-NAME+Indo. Analysis of the AUC for ANG II responses demonstrated that blockade of NO increased ANG II-mediated contraction and the magnitude of this effect did not vary significantly between genotypes (Fig. 7, A–D). In contrast, blockade of prostanoid production with Indo significantly augmented ANG II-mediated contraction in arteries of Rln+/−/− mice (P<0.006), whereas it had no effect in Rln+/++/++ mice (Fig. 7 D). Blockade with l-NAME+Indo augmented AUC of ANG II contraction to a similar extent to that of l-NAME alone (both genotypes) and Indo alone (Rln+/++/++).

To investigate whether the modified ANG II contraction in late-pregnant Rln−/− mice was dependent on the endothelium, mesenteric arteries were either denuded or left intact before exposure to ANG II. ANG II-evoked contraction was not significantly altered by removal of the endothelium in arteries of either genotype (Fig. 7 E), demonstrating that the enhanced vascular responsiveness to ANG II in Rln−/− mice is independent of the endothelium.

DISCUSSION

Mesenteric arteries undergo marked functional adaptation during pregnancy, characterized by suppression of agonist-induced contraction. The key finding in this study was the failure of mesenteric arteries, but not uterine arteries, of Rln−/− mice to adapt appropriately to pregnancy. Specifically, contraction to ANG II was enhanced compared with wild-type counterparts. This was not due to elevated ROS generation in Rln−/− mice or altered expression of ANG II receptors. An important finding was that the enhanced ANG II-mediated contraction response in Rln−/− mice was partly dependent on the endothelium.
contraction in late-pregnant Rln−/− mice was not dependent on the endothelium, reflecting inherent differences in smooth muscle responsiveness. Furthermore, the diminished role of basal vasodilator prostanoids (likely vascular smooth muscle-derived) in late-pregnant Rln−/− mice appears to contribute to the increased responsiveness to ANG II.

In nonpregnant females, relaxin deficiency is without effect on mesenteric artery responsiveness to vasoconstrictors. This is in contrast to the mesenteric artery of male Rln−/− mice, which has increased sensitivity to both PE and U46619, attributed to impairments in NO and vasodilator prostanoid pathways (26). Premenopausal women are better protected against cardiovascular disease than men (5), and this is, at least in part, because of the hormone estrogen and its ability to stimulate generation of the vasodilator molecules NO and prostacyclin (2). Ovulation is not affected in female Rln−/− mice, so we suggest that estrogen levels may be sufficient to compensate for the lack of relaxin in estrus.

In pregnant Rln+/+ mice, circulating relaxin levels increase markedly and correspond to a reduction in responsiveness to vasoconstrictors, particularly ANG II. This did not occur in late-pregnant Rln−/− mice. Diminished responses to ANG II have been demonstrated in a range of arteries, including the mesenteric and uterine arteries of early- and late-pregnant rats, Table 2.

Table 2. Reactivity of main uterine arteries of Rln+/+ and Rln−/− mice at estrus and on D17.5 of pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Rln+/+</th>
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<tr>
<td></td>
<td>Estrus</td>
<td>D17.5</td>
<td>Estrus</td>
<td>D17.5</td>
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<tr>
<td>ET1 AUC</td>
<td>168.3 ± 11.4</td>
<td>122.7 ± 6.6</td>
<td>179.3 ± 20.9</td>
<td>126.0 ± 12.2</td>
</tr>
<tr>
<td>pEC50 U46619</td>
<td>8.8 ± 0.2</td>
<td>8.2 ± 0.1*</td>
<td>8.6 ± 0.2</td>
<td>8.2 ± 0.1</td>
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<tr>
<td>PE AUC</td>
<td>339.3 ± 53.8</td>
<td>268.1 ± 11.9</td>
<td>308.1 ± 12.6</td>
<td>275.0 ± 19.6</td>
</tr>
<tr>
<td>pEC50 ANG II</td>
<td>6.8 ± 0.3</td>
<td>8.3 ± 0.1</td>
<td>8.6 ± 0.2</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>AUC</td>
<td>358.2 ± 11.2</td>
<td>378.0 ± 9.5</td>
<td>371.1 ± 18.3</td>
<td>368.8 ± 17.3</td>
</tr>
<tr>
<td>pEC50</td>
<td>8.9 ± 0.1</td>
<td>9.5 ± 0.2*</td>
<td>9.0 ± 0.2</td>
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Values are expressed as means ± SE. *Significantly different compared with estrus within genotype.

Fig. 5. Relaxin deficiency has no effect on vascular ANG II-related gene expression in late pregnancy. Quantitative PCR analysis of ANG II receptor 1a (Agtr1a), angiotensin II receptor type 2 (Agtr2), and regulator of G protein signaling 5 (Rgs5) expression in mesenteric arteries from mice at estrus (A, C, and E) and on D17.5 of pregnancy (B, D, and F) in (○) Rln+/+ and (△) Rln−/− mice (n = 7 per group). Horizontal bars indicate mean values.
respectively (17), as well as the renal circulation in mid-term pregnant rats (39). Consistent with our data, Pulger et al. (41) reported increased responsiveness of the mouse uterine artery to ANG II in pregnancy, which was likely balanced by increased AT2 receptor-mediated activity to maintain enhanced artery vasodilatation. There is good evidence that NO mediates, in part, the reduction in the systemic pressor response to ANG II (34) but not the attenuated renal response to ANG II (39) in pregnant rats. Chronic administration of porcine relaxin to conscious female rats attenuates renal vasoconstriction produced by ANG II, linking relaxin to decreased responsiveness to ANG II in vivo (10). Furthermore, acute relaxin treatment for 6 h increases global arterial compliance and decreases systemic vascular resistance without changing mean arterial pressure (12). Chronic administration of relaxin for 2 wk also reverses ANG II-induced hypertension in male Sprague-Dawley rats (43). This was prevented by treatment with l-NAME, demonstrating that the effects of relaxin were dependent on NO synthase. Acute and 24-h relaxin administration to cultured renal mesangial cells of virgin rats reduced the intracellular calcium influx caused by ANG II (4). This further supports our idea that relaxin is a novel regulator of ANG II action.

In many vascular studies, relaxin appears to act through NO signaling (20, 32, 38, 43), so we predicted that the functional phenotype in the mesenteric artery of pregnant Rln−/− mice would be associated with a reduction in the basal NO. However, the enhanced ANG II-mediated contraction was not driven by an underlying deficit in NO signaling. This is similar to our finding that endothelial dysfunction in mesenteric arteries of male Rln−/− mice was not related to alterations in basal NO (25). In the absence of an NO deficit, we explored other options to explain why mesenteric arteries from pregnant Rln−/− mice retained the same responsiveness to ANG II as in virgin animals. One possible mechanism could involve altered expression of receptors, either the proconstrictor AT1 receptor or the vasodilator AT2 receptor (36). AT2 receptors in uterine artery and aorta are increased in pregnancy (42, 46) and AT2 receptor-mediated vasodilatation is an important contributor to the attenuated constrictor response to ANG II in these arteries in pregnant rats and mice (41, 46). There were no differences in receptor expression between the genotypes in the mesenteric artery. This implies that relaxin deficiency does not compromise AT receptor expression. RGS5 regulates ANG II signaling at a post-transcriptional level (16); RGS5 knockout mice are hypertensive, and their mesenteric arteries display increased sensitivity to ANG II in late pregnancy (18). Thus, decreases in Rgs5 in our pregnant Rln−/− mice could explain the enhanced contraction to ANG II. Interestingly, we observed a reduction in Rgs5 expression in the mesenteric arteries of both Rln+/+ and Rln−/− mice in late pregnancy but no difference between the genotypes, so this does not account for the increased ANG II sensitivity in the mesenteric arteries of pregnant Rln−/− mice.

Enhanced constrictor responses to ANG II are partially attributed to increased ROS activation in human omental arteries from preeclamptic women (33). Subcutaneous relaxin infusion lowers blood pressure in ANG II-induced hypertensive rats through a mechanism involving a reduction in oxidative stress (43). We considered that the enhanced constriction to ANG II in the mesenteric arteries of Rln−/− mice was underpinned by elevated ROS generation. Furthermore, we have previously demonstrated that superoxide production was significantly increased in the aorta of male Rln−/− mice (35). In the current study, there was insufficient tissue available to measure ROS generation, so we incubated arteries with ROS scavengers and assessed constriction to ANG II. There were no differences in ANG II dose-response curves for mesenteric arteries from Rln+/+ and Rln−/− mice, so these findings suggest that increased ROS generation is unlikely to account for
the impaired adaptation of the mesenteric artery to ANG II in pregnant Rln"-/- mice.

The endothelium is an important regulator of vascular tone in pregnancy (3). Blocking cyclooxygenase activity enhanced ANG II contraction in mesenteric arteries of our pregnant Rln"+/+ mice, but not Rln"-/- mice. This suggests that in arteries of Rln"+/+ mice, basal production of vasodilator prostanoids serve to suppress contraction to ANG II. Consistent with this, PGI2 receptor expression and sensitivity to the PGI2 analog iloprost are reduced in the aorta of male Rln"-/- mice (35). Furthermore, relaxin treatment in male rats significantly increases PGI2-mediated relaxation in the mesenteric arteries as area under the curve (AUC) for control (C) and difference responses (D) to control after incubation with L-NAME, Indo, and L-NAME+Indo from Rln"+/+ (solid bars) and Rln"-/- mice (open bars) on day 17.5 of pregnancy (n = 6 or 7 per group). *P < 0.05 treatment between genotypes. E: concentration-response curves to ANG II in endothelium-intact (solid symbols) and denuded (open symbols) mesenteric arteries isolated from Rln"+/+ (●) and Rln"-/- (●) mice on day 17.5 of pregnancy (n = 7 per group).

Perspectives and Significance

This study has uncovered an important functional connection between circulating relaxin and the vascular responses to ANG II in mesenteric arteries in pregnancy. In the absence of relaxin (in Rln"-/- mice), the normal pregnancy adaptation of attenuated ANG II-mediated constriction does not occur. In contrast to previous studies, we have shown this is not underpinned by an impaired endothelium-dependent NO contribution, but is associated with a deficit in smooth muscle-derived vasodilator prostanoids. The physiological consequences of this enhanced ANG II sensitivity in the mesenteric artery were not established in this study, but might include increased blood pressure. Our data are directly relevant to better understanding...
why the severity of preeclampsia in pregnant women might be related to low levels of relaxin. We suggest that in the absence of relaxin, the mesenteric vasculature loses its ability to down-regulate its responsiveness to vasoconstrictors and, thus, predisposes women to hypertensive disorders of pregnancy, including preeclampsia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


