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Article

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Relaxin is essential for renal vasodilation during pregnancy in conscious rats

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Marked vasodilation in the kidney and other nonreproductive organs is one of the earliest maternal adaptations to occur during pregnancy. Despite the recognition of this extraordinary physiology for over four decades, the gestational hormone responsible has remained elusive. Here we demonstrate a key role for relaxin, a member of the IGF family that is secreted by the corpus luteum in humans and rodents. Using a gravid rodent model, we employ two approaches to eliminate relaxin or its biological activity from the circulation: ovariectomy and administration of neutralizing antibodies. Both abrogate the gestational elevation in renal perfusion and glomerular filtration, as well as preventing the reduction in myogenic reactivity of isolated, small renal arteries. Osmoregulatory changes, another pregnancy adaptation, are also abolished. Our results indicate that relaxin mediates the renal vasodilatory responses to pregnancy and thus may be important for maternal and fetal health. They also raise the likelihood of a role for relaxin in other cardiovascular changes of pregnancy, and they suggest that, like estrogen, relaxin should be considered a regulator of cardiovascular function.

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Introduction

Among the most striking changes seen in biology are those associated with pregnancy. In particular, profound vasodilation of nonreproductive organs including the kidney epitomizes the maternal cardiovascular adaptation to early gestation in women. Cardiac output, global arterial compliance, effective renal plasma flow (ERPF), and GFR rise from 30% to 80%, while vascular resistances plummet and blood pressure declines modestly (refs. 1, 2; and reviewed in ref. 3). These alterations begin immediately after conception, peak by the end of the first or beginning of the second trimester, and persist throughout gestation. It is likely that the circulations of nonreproductive organs such as the kidney serve as arteriovenous shunts during early gestation. Thus, ventricular afterload falls, which initiates the enormous increase in cardiac output and, subsequently, the expansion of plasma volume — maternal adaptations associated with healthy pregnancies. Furthermore, pressor response to administration of angiotensin II and vascular reactivity to infusion of norepinephrine become attenuated. Insight into the mechanisms responsible for these vasodilatory phenomena may be particularly critical, since in preeclampsia, the attenuation of pressor responsiveness to angiotensin II, the reduced vascular reactivity to norepinephrine, and the systemic and renal vasodilation are compromised (3).

Although 17β-estradiol has been traditionally viewed as the uterine and systemic vasodilator of pregnancy (4, 5), this hormone has little, if any, effect on the renal circulation (5–8), which markedly vasodilates so early in pregnancy. Progesterone may have limited capacity to vasodilate the renal circulation (6, 9, 10); however, an alternative possibility is that the pregnancy protein hormones are involved. Relaxin is an approximately 6-kDa protein secreted by the corpus luteum during human gestation (reviewed in ref. 11). Stimulated by the luteotrophic hormone human chorionic gonadotrophin, serum levels of relaxin increase immediately after conception (11) corresponding to the gestational rise in ERPF and GFR (3). Relaxin also circulates, albeit at lower levels, in the luteal phase of the menstrual cycle (11) and is associated with a 20% increase in ERPF and GFR at that time (e.g., see ref. 12). The hormone is temporally linked to another early pregnancy adaptation — osmoregulatory changes (13). In gravid rats, relaxin is secreted by the corpora lutea with serum levels first detectable on gestational day 8 (11).

The primary objective of the present investigation was to test whether endogenous relaxin mediates the renal vasodilation and hyperfiltration of pregnancy in conscious rats. We investigated midterm pregnancy (days 11–14) when GFR and ERPF are at peak levels during gestation in this species (14). Another goal was to determine whether the hormone mediates the reduction in myogenic reactivity of isolated, small renal arteries typically observed during rat gestation (15). Finally, we also set out to determine whether relaxin is responsible for the gestational changes in osmoregulation in this animal model (14).

Methods

Animal preparation. All procedures were approved by the Institutional Care and Use Committee of the Magee-Women's Research Institute or of the University of New Mexico School of Medicine. Long-Evans female rats of 13–18 weeks of age were purchased from Harlan Sprague Dawley Inc. (Indianapolis, Indiana, USA or Frederick, Maryland, USA) and fed a PROLAB RMH 2500 or 2000 diet (PMI Nutrition International Inc., Brentwood, Missouri, USA). They were maintained on a 12/12-hour light/dark cycle in the Research Resource Facilities. The rats were habituated to the experimental conditions, and then instrumented with chronic arterial, venous, and bladder catheters as previously described (14). After 5–10 days of surgical recovery, the rats were housed with males. The presence of spermatozoa in the vaginal lavage marked day 0 of gestation. Implantation occurs on day 5 or 6 in the rat, and gestation lasts 21–22 days.

Administration of MCA1 or MCAF antibodies. Neutralizing mAb against rat relaxin (MCA1) or control mAb against fluorescein (MCAF) was administered daily between 12 and 4 pm from day 8 to day 14 of pregnancy. Each dose of 5.0 mg in 0.5 ml saline was infused over 5 minutes into the venous catheter (16, 17). The dose, route, and timing of antibody administration were the same as those previously reported to eliminate a variety of relaxin-induced changes in the reproductive tract during pregnancy (e.g., refs. 16, 17). On the morning of gestational days 11 and 14, 200 μl of blood was taken from the arterial catheter at the beginning of the experiment for measurement of plasma sodium concentration and osmolality. Next, GFR and ERPF were measured by the renal clearances of inulin and para-aminohippurate, respectively, and mean arterial pressure (MAP) was monitored continuously throughout the experiment as previously described (14). Four renal clearances of 0.5 hours each were obtained for inulin and para-aminohippurate, and the results were averaged. After the in vivo experimentation on day 14, one kidney was removed and given to J. Novak or R.J. Ramirez in a blinded fashion for analysis of myogenic reactivity of small renal arteries in vitro using a pressurized arteriograph (ref. 15, and see below). To exclude the possibility that immune complexes were formed and deposited in the glomerulus during administration of the MCA1 antibody, the other kidney was processed for transmission electron microscopy — a sensitive technique for detection of immune complex deposition. Additional rats were surgically prepared, but not mated, and studied concurrently with the pregnant rats (virgin controls).

Ovariectomy. Using another experimental approach, rats were ovariectomized on gestational day 8 to eliminate circulating relaxin and implanted with silastic tubes containing 17β-estradiol (2 μg) and progesterone (240 mg) to maintain pregnancy. Other rats were sham-ovariectomized. These rats were investigated on gestational days 12–14 in a manner comparable to that of the animals receiving the antibodies as described above. The doses of 17β-estradiol and progesterone as well as the method of administration were previously shown to produce serum levels of 25–35 pg/ml and 55–65 ng/ml, respectively, which were not significantly different from those of sham-operated pregnant rats of comparable gestational age (11–14 days) (18).

Myogenic reactivity of small renal arteries. After assessment of renal function in the conscious rats as described above, the myogenic reactivity of small renal arteries isolated from the same rats was investigated. For the ovariectomy model, an additional two each of the ovariectomized steroid-replaced and sham-operated pregnant rats were prepared only for study of myogenic reactivity. One kidney was removed and placed in an ice-cold HEPES-buffered physiological saline solution — a modified Krebs buffer composed of sodium chloride 142 mmol/l, potassium chloride 4.7 mmol/l, magnesium sulfate 1.17 mmol/l, calcium chloride 2.5 mmol/l, potassium phosphate 1.18 mmol/l, HEPES 10 mmol/l, and glucose 5.5 mmol/l — and maintained at a pH of 7.4 at 37°C (15). Segments of renal interlobar artery branches were dissected free of surrounding tissue (unpressurized inner diameter, 100–200 μm). An arterial segment was then transferred to the isobaric arteriograph (Living Systems Instrumentation, Burlington, Vermont, USA) and mounted on two microcannulae suspended in the chamber. After residual blood was washed from the lumen of the vessel, the distal cannula was occluded to prevent flow. The proximal cannula was attached to a pressure transducer, a pressure servocontroller, and a peristaltic pump. The servocontroller maintained a selected intraluminal pressure, which could be rapidly changed in a stepwise manner. The arteriograph was placed on the stage of an inverted microscope with a video camera to provide a video image of the vessel. Arterial diameter was obtained by an electronic dimension analyzing system (Living Systems Instrumentation). The artery was then constricted to 80% of its initial diameter at 60 mmHg with phenylephrine. After the new diameter was recorded, the intraluminal pressure was rapidly increased in a stepwise manner to 80 mmHg. The pressure was maintained at 80 mmHg until the artery stabilized at a new diameter (~4 minutes). This new diameter was recorded, and then the

pressure was returned to 60 mmHg and the artery allowed to stabilize again before the entire process was repeated for a total of three pressure steps. The data were expressed as percent change in diameter at 80 mmHg compared to the diameter at 60 mmHg. The three responses from each vessel were averaged.

Estrogen and progesterone assays. Serum 17β-estradiol and progesterone concentrations were measured in the ovariectomized steroid-replaced and the sham-operated pregnant rats, in order to document the adequacy of the replacement regimen. The assays were conducted in the Assay Core of the Center for Research in Reproduction at the University of Pittsburgh School of Medicine, and the methodologies have been previously reported (19, 20). Briefly, the minimum detectable concentrations of progesterone and 17β-estradiol were 75 pg/ml and 0.625 pg/ml, respectively. The interassay coefficient of variation was 13.3% (50 assays at 57% binding) and 18.7% (20 assays at 64% binding) for the progesterone and 17β-estradiol assays, respectively. The corresponding intra-assay coefficient of variation was 10.5% at 67% binding and 13.9% at 64% for the two assays. In pooled serum of ovariectomized rats, progesterone and 17β-estradiol concentrations were low — 69 and 1.1 pg/ml, respectively. These "blank" values were subtracted from all standard curve and sample concentrations. For additional quality control, serum from ovariectomized animals spiked with known concentrations of the two steroids were routinely tested in each assay.

Transmission electron microscopy. To exclude the possibility that immune complexes were deposited in the glomerulus during administration of relaxin-neutralizing antibody to pregnant rats, thereby reducing GFR and ERPF, we performed transmission electron microscopy (TEM). TEM is a sensitive method to detect immune complex deposition in the kidney. Slices of rat kidneys were fixed by immersion in 2.5% glutaraldehyde/2% *p*-formaldehyde in sodium cacodylate buffer for at least 3 hours, and then rinsed in 1% sodium cacodylate buffer (pH 7.4). The specimens were next minced into 1-mm cubes and post-fixed in 1% aqueous osmium tetroxide for 1 hour at 4°C. The tissues were dehydrated in ascending alcohols from 70% through 100%, and subjected to two 0.5-hour changes of propylene oxide. Three 1-hour infiltration steps — 1:2 and 2:1 propylene oxide/EMbed 812-Araldite resin mixtures and pure EMbed 812-Araldite (EMbed-812 and Araldite 502; Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) — preceded polymerization in Beem capsules for 24 hours at 60°C. One-micron sections were cut and stained with 1% toluidine blue O in 1% sodium borate. Suitable areas were selected, and ultrathin sections were cut, mounted on copper grids, and stained with saturated alcoholic uranyl acetate and lead citrate. Sections were examined on a Philips CM12 electron microscope (FEI Co., Hillsboro, Oregon, USA) by two renal pathologists. All procedures were conducted in Anatomic Pathology, Department of Electron Microscopy, University of Pittsburgh School of Medicine.

Statistical analyses. Data were analyzed by two-factor randomized block design ANOVA followed by comparison of group means by contrasts, or by unpaired *t* tests (21, 22). $P < 0.05$ was taken to be significant.

Results

To test the role of endogenous relaxin in renal and osmoregulatory changes of pregnancy, the neutralizing MCA1 or control MCAF mAb (16, 17) was administered daily from days 8–14 of pregnancy. The relaxin-neutralizing antibody completely abrogated the gestational increase in GFR and ERPF, as well as the reduction in effective renal vascular resistance (ERVR = MAP ÷ ERPF/1.0-hematocrit) on gestational days 11 (Figure 1, a–c) and 14 (Figure 2, a–c). Furthermore, the typical reduction of myogenic reactivity observed in small renal arteries from pregnant rats ex vivo (15) was also abolished by the relaxin-

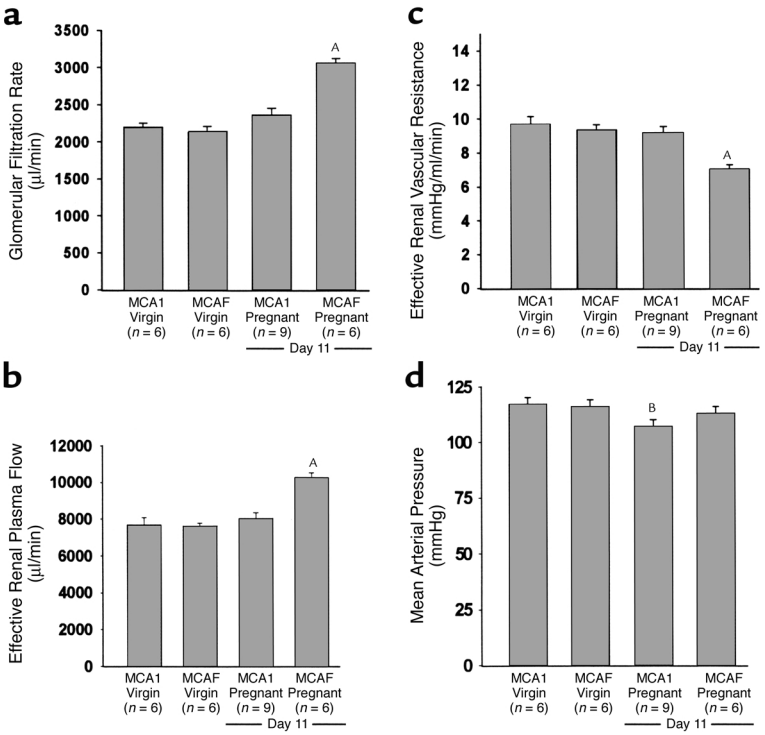

Figure 1
Renal function in conscious virgin and day 11 pregnant rats treated with rat relaxin-neutralizing antibody (MCA1) or control antibody (MCAF). (**a**) GFR. (**b**) ERPF. (**c**) ERVR. (**d**) MAP. $^{A}P < 0.01$ vs. other groups; $^{B}P < 0.05$ vs. MCA1 and MCAF virgin.

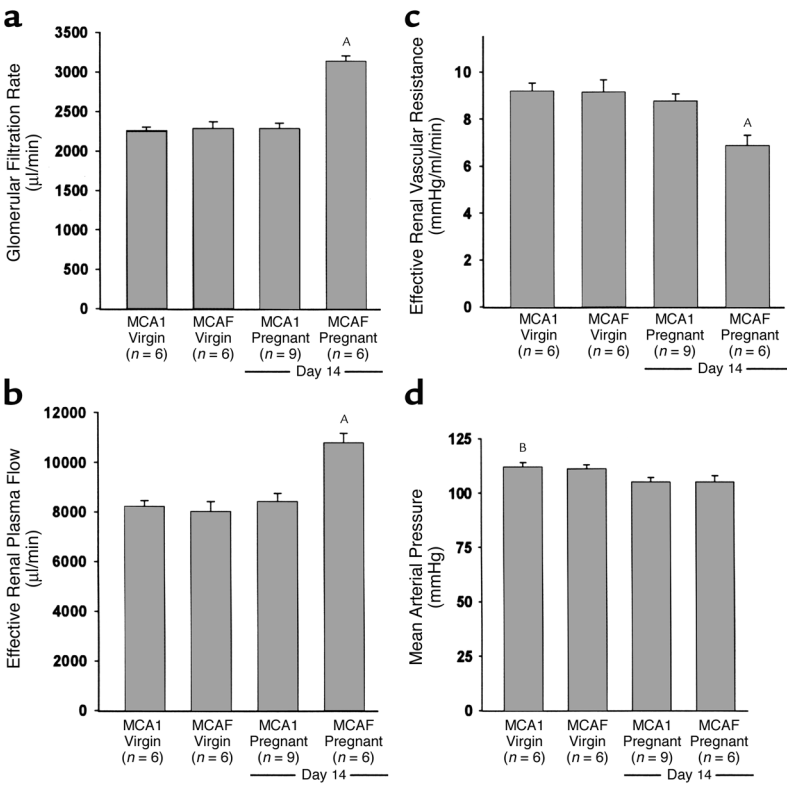

Figure 2
Renal function in conscious virgin and day 14 pregnant rats treated with rat relaxin-neutralizing antibody (MCA1) or control antibody (MCAF). (**a**) GFR. (**b**) ERPF. (**c**) ERVR. (**d**) MAP. $^{A}P < 0.01$ vs. other groups; $^{B}P \leq 0.05$ vs. MCA1 and MCAF pregnant.

neutralizing antibody (Figure 3a). In contrast, the small reduction in MAP that can be observed at midgestation in the gravid rat (14) was not blocked by the relaxin-neutralizing antibody (Figures 1d and 2d). Thorough examination by TEM of at least three glomeruli each from the pregnant rats treated either with the relaxin-neutralizing antibody (n = 3 rats) or control antibody (n = 1) was performed (×5,600 to ×28,000 magnification). We observed no immune complex deposition in the glomeruli or elsewhere in the renal cortex, thereby precluding this potential side effect as an explanation for the abolition of renal circulatory changes during pregnancy by the relaxin-neutralizing antibody (data not shown).

The usual decreases in plasma osmolality and sodium concentration found during pregnancy (14) were inhibited by MCA1 antibody on both gestational days 11 and 14 (Table 1). Previously, the growth of the cervix during rat pregnancy has been attributed to relaxin (17, 18). Thus, as confirmation of the efficacy of the relaxin-neutralizing antibody in the current study, we measured cervical wet weight. The relaxin-neutralizing antibody prevented cervical growth in the pregnant animals (Table 1). For the MCAF and MCA1 antibody treatment groups, respectively, there were 13.8 ± 0.9 and 13.1 ± 0.6 fetuses on gestational day 14 (P = not significant [NS]). Of these, 0.9 ± 0.4 and 2.2 ± 0.6, respectively, were being absorbed ($P < 0.05$).

Serum levels of progesterone were 46.0 ± 3.7 and 42.1 ± 4.8 ng/ml, respectively, in the MCAF- and MCA1-treated pregnant rats (P = NS). The 17β-estradiol concentrations were correspondingly 21.1 ± 4.0 and 25.9 ± 3.7 pg/ml (P = NS).

Using another experimental approach, rats were ovariectomized on gestational day 8 to eliminate circulating relaxin and the pregnancy was maintained with exogenous 17β-estradiol and progesterone (18). Serum levels of 17β-estradiol were 32.2 ± 4.5 and 20.3 ± 3.7 pg/ml in the sham-operated and ovariectomized steroid-replaced rats, respectively (P = 0.07). Despite a low value for 17β-estradiol of 7.9 pg/ml in one of the rats included in the mean for the latter group, she carried 16 fetuses and 2 absorptions — comparable to the other rats (see below). As it happened, this rat was one of those used only for the study of myogenic reactivity and not for study of renal function. Serum levels of progesterone were 59.1 ± 4.2 and 65.5 ± 3.8 ng/ml in the sham-operated and ovariectomized steroid-replaced rats, respectively (P = NS).

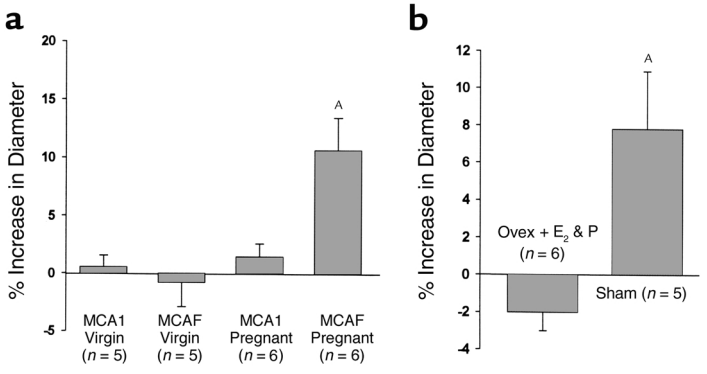

Figure 3
Myogenic reactivity of small renal arteries. (**a**) Virgin and day 12 to 14 pregnant rats treated with rat relaxin-neutralizing antibody (MCA1) or control antibody (MCAF). (**b**) Pregnant rats were either ovariectomized (Ovex) on gestational day 8 and replaced with 17β-estradiol (E_2) and progesterone (P) or sham-ovariectomized. $^{A}P < 0.01$ vs. other group(s).

Table 1
Plasma osmolality, sodium concentration, and cervical wet weight

Gestational day 11	MCA1 virgin (n = 6 rats)	MCAF virgin (n = 6 rats)	MCA1 pregnant (n = 9 rats)	MCAF pregnant (n = 6–8 rats)
Plasma osmolality (mOsm/kg H_2O)	305 ± 2	300 ± 3	303 ± 1	291 ± 2[A]
Plasma sodium (mmol/l)	140 ± 2	140 ± 2	143 ± 1	135 ± 2[A]
Gestational day 14				
Plasma osmolality (mOsm/kg H_2O)	301 ± 3	301 ± 5	304 ± 2	290 ± 3[A]
Plasma sodium (mmol/l)	141 ± 3	142 ± 3	143 ± 1	135 ± 1[A]
Cervical wet weight (mg)	35 ± 4	34 ± 5	38 ± 4	53 ± 6[A]

Data are shown as mean ± SEM. MCA1, rat neutralizing mAb; MCAF, mAb vs. fluorescein (control). [A]$P < 0.05$ vs. all other groups.

Complete abrogation of the renal circulatory and osmoregulatory changes (Table 2), as well as of the gestational reduction in myogenic reactivity of small renal arteries ex vivo (Figure 3b), was observed on gestational days 12–14 in the ovariectomized steroid-replaced pregnant rats, comparable to the results obtained using relaxin-neutralizing antibodies. For the sham-operated and ovariectomized groups, respectively, there were 13.0 ± 0.6 and 13.1 ± 0.8 fetuses on gestational days 12–14. Of these, 0.6 ± 0.4 and 2.2 ± 0.8, respectively, were being absorbed ($P < 0.06$).

Discussion

Current understanding of the vasodilatory mechanisms of normal pregnancy has been facilitated by using animal models. The chronically instrumented, conscious pregnant rat manifests renal, cardiovascular, and endocrine changes remarkably similar to those of gravid women (14, 23–25). In addition to renal vasodilation and hyperfiltration (14), small arteries from gravid rats show diminished myogenic reactivity in vitro (15). Pregnant rats also manifest increased biosynthesis of nitric oxide (NO) (26) and its second messenger, cGMP (27). NO mediates the renal circulatory changes of pregnancy in the gravid rat model (28), as well as the reduced myogenic reactivity of small renal arteries isolated from gravid rats in vitro (15). Endothelin (ET) via the ET_B receptor subtype on the endothelium drives the NO-dependent renal vasodilation and hyperfiltration in pregnant rats (29), as well as the NO-dependent reductions in myogenic reactivity of small renal arteries in vitro (15).

We questioned which pregnancy hormone(s) stimulates the ET/NO vasodilatory pathway in the renal circulation during gestation, and we considered relaxin as a likely candidate (ref. 30; and see Introduction, above). Further rationale for the proposed role of relaxin in the renal circulatory changes of pregnancy is that chronic administration of the hormone reduces blood pressure and vasoconstrictor responses in the mesenteric circulation of spontaneously hypertensive rats, and acute treatment increases coronary blood flow and reduces platelet aggregation via NO and cGMP (31, 32). We found that both the renal and the osmoregulatory effects of chronic relaxin administration to nonpregnant rats resemble the physiological changes of pregnancy in several respects: (a) marked increases in ERPF and GFR with a mediatory role for NO, (b) attenuation of renal vasoconstriction to angiotensin II, and (c) reduction in plasma sodium concentration and osmolality (30). Interestingly, the renal response persists in ovariectomized animals, indicating that an intermediary or permissive role for sex steroids is not required (30), in contrast to many of the effects of relaxin on the female reproductive tract (11). Surprisingly, comparable renal and osmoregulatory responses to relaxin administration are observed in conscious male rats (33). Analogous to midterm pregnancy (29), relaxin-induced renal vasodilation and hyperfiltration in nonpregnant rats are also mediated by ET through the endothelial ET_B receptor subtype and NO (33). The molecular mechanisms underlying the ET/NO-mediated renal vasodilation of pregnancy by relaxin is currently under investigation.

The best (if not only) approach for testing the physiologic role of a hormone is to block production or inhibit action of the hormone, as we did in the current work. Unfortunately, the relaxin receptor has not been identified, which precludes the more traditional route of receptor antagonism for assessing the physiological role of relaxin in pregnancy. Nevertheless, results from the two methodological approaches used here provide strong support for a critical role of relax-

Table 2
Renal function, plasma osmolality, and sodium concentration on gestational days 12–14 in ovariectomized steroid-replaced and sham-ovariectomized rats

	GFR (μl/min)	ERPF (μl/min)	ERVR (mmHg/ml/min)	P_{osm} (mOsm/kg H_2O)	P_{Na+} (mmol/l)	MAP (mmHg)
Ovex/E_2-P replacement (n = 3 rats)	2505 ± 81	7293 ± 706	9.18 ± 0.59	300 ± 2	143 ± 1	110 ± 3
Sham ovex (n = 4 rats)	3290 ± 184[A]	10,795 ± 199[A]	6.65 ± 0.13[A]	289 ± 2[A]	135 ± 2[A]	115 ± 1

Data are presented as mean ± SEM. P_{osm}, plasma osmolality; P_{Na+}, plasma sodium concentration; ovex, ovariectomy; E_2, 17β-estradiol; P, progesterone. [A]$P < 0.02$ vs. ovex/E_2-P replacement.

in in the renal circulatory and osmoregulatory changes of pregnancy. In fact, we were surprised by the complete (rather than partial) nature of the inhibition imposed by the relaxin-neutralizing antibody or ovariectomy on renal circulatory and osmoregulatory changes during pregnancy. Apparently, contributing or compensatory mechanisms are lacking, which underscores the fundamental and essential role of relaxin in these maternal adaptations at least during midpregnancy in the rat.

Interestingly, the small reduction in MAP at midgestation was not inhibited by the relaxin-neutralizing antibody. This finding is consistent with the work of Ahokas and colleagues, who showed that the larger reduction in MAP observed at late gestation was not attenuated in ovariectomized steroid-replaced rats (34). The kidneys receive about 25% of the cardiac output and are major determinants of systemic vascular resistance; thus, we anticipated an attenuation of the gestational decline in MAP during MCA1 administration, because the gestational decline in ERVR was inhibited by this experimental intervention. In order to resolve this apparent discrepancy, simultaneous measurements of cardiac output and MAP are needed to calculate systemic vascular resistance.

Considerable evidence exists for interactions between 17β-estradiol and relaxin. First, many of the effects of relaxin in the female reproductive tract require the permissive action of 17β-estradiol or are potentiated by the hormone (11). Second, 17β-estradiol derived from intraovarian aromatization of androgens is involved in the secretion of relaxin from the corpus luteum by luteotrophic placental factors (35, 36). Third, relaxin may stimulate uterine edema in the rat ultimately by phosphorylating and activating an estrogen receptor (37). Thus, potential interaction between 17β-estradiol or its receptors and relaxin in the kidney warrants consideration.

Although we cannot completely exclude the possibility of such an interaction in the renal circulation, the available evidence is not supportive. We previously showed that the renal vasodilation and hyperfiltration in response to relaxin are similar in ovariectomized and sham-ovariectomized nonpregnant rats (30). Furthermore, relaxin produces similar or even greater increases of GFR and ERPF in male rats (33). These two observations suggest that the renal action of relaxin does not require 17β-estradiol, at least in the nonpregnant state, insofar as 17β-estradiol levels are extremely low in ovariectomized female and in male rats. Because 17β-estradiol alone has little, if any, effect on the renal circulation (5–8), and baseline GFR and ERPF are comparable in ovariectomized and sham-ovariectomized rats (30), it seems unlikely that relaxin is ultimately activating an estrogen receptor (as it may in the uterus; see above) to produce renal vasodilation and hyperfiltration.

Finally, the current work also argues against an interaction between 17β-estradiol and relaxin in the renal circulation during pregnancy. That is, either removal of relaxin from the circulation by ovariectomy while maintaining pregnancy levels of 17β-estradiol with exogenous administration, or specific neutralization of endogenous rat relaxin during pregnancy using mAb's that did not affect circulating levels of 17β-estradiol, completely abolished the renal circulatory adaptations to pregnancy.

The cardiovascular system during normal pregnancy is the epitome of health, typified by low systemic vascular resistance, modestly reduced blood pressure, increased arterial compliance, and robust endothelial function (3). Moreover, hypertension later in life is less frequent in women who have normal pregnancy(s) (38). Thus, we believe that discovery of the pregnancy hormones responsible for cardiovascular changes in pregnancy is a priority, as these hormones may serve as new therapeutic agents for increased vascular resistance and arterial stiffness that occur with aging and hypertension in both men and women. In this regard, we hypothesize that the pregnancy hormone relaxin holds tremendous potential. Whether the vasodilatory role of relaxin in rats can be extrapolated to humans requires additional investigation (although part of the rationale for the present study was based on observations in humans). Nevertheless, it is exciting to further contemplate a potential therapeutic role for relaxin in renal disease, not only with respect to its newly described vasodilatory properties (30), but also in light of its well-known matrix-degrading attributes (39).

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