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RESEARCH ARTICLE | *Renal Hemodynamics*

Effects of serelaxin on renal microcirculation in rats under control and high-angiotensin environments

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Shao W, Rosales CB, Gonzalez C, Prieto MC, Navar LG. Effects of serelaxin on renal microcirculation in rats under control and high-angiotensin environments. *Am J Physiol Renal Physiol* 314: F70–F80, 2018. First published October 4, 2017; doi:10.1152/ajprenal.00201.2017.—Serelaxin is a novel recombinant human relaxin-2 that has been investigated for the treatment of acute heart failure. However, its effects on renal function, especially on the renal microcirculation, remain incompletely characterized. Our immunoexpression studies localized RXFP1 receptors on vascular smooth muscle cells and endothelial cells of afferent arterioles and on principal cells of collecting ducts. Clearance experiments were performed in male and female normotensive rats and Ang II-infused male rats. Serelaxin increased mean arterial pressure slightly and significantly increased renal blood flow, urine flow, and sodium excretion rate. Group analysis of all serelaxin infusion experiments showed significant increases in GFR. During infusion with subthreshold levels of Ang II, serelaxin did not alter mean arterial pressure, renal blood flow, GFR, urine flow, or sodium excretion rate. Heart rates were elevated during serelaxin infusion alone ($37 \pm 5\%$) and in Ang II-infused rats ($14 \pm 2\%$). In studies using the in vitro isolated juxtamedullary nephron preparation, superfusion with serelaxin alone (40 ng/ml) significantly dilated afferent arterioles (10.8 ± 1.2 vs. 13.5 ± 1.1 μm) and efferent arterioles (9.9 ± 0.9 vs. 11.9 ± 1.0 μm). During Ang II superfusion, serelaxin did not alter afferent or efferent arteriolar diameters. During NO synthase inhibition (L-NNA), afferent arterioles also did not show any vasodilation during serelaxin infusion. In conclusion, serelaxin increased overall renal blood flow, urine flow, GFR, and sodium excretion and dilated the afferent and efferent arterioles in control conditions, but these effects were attenuated or prevented in the presence of exogenous Ang II and NO synthase inhibitors.

afferent arteriole; efferent arteriole; relaxin family peptide receptors; renal function

INTRODUCTION

Relaxin is a peptide hormone of the insulin/relaxin family of structurally related hormones that is secreted by the corpus luteum of the ovary and circulates in the blood during pregnancy in humans, rats, and mice (43, 44). Relaxin is also produced locally in blood vessels of males and females, where it probably acts as a paracrine or autocrine factor (32, 34). Relaxin is widely recognized for mediating the essential renal and systemic hemodynamic adaptations in early pregnancy through direct actions on the maternal vasculature (6, 7). Serelaxin is a recombinant human relaxin-2 that has been investigated for the treatment of acute heart failure (31, 51). In heart failure, the renin-angiotensin system is activated, and the plasma and tissue Ang II levels are elevated (38, 49). Short-term or long-term administration of relaxin can attenuate the renal vasoconstrictor response to Ang II (13). A previous study demonstrated that the vasodilatory effects of relaxin increased nitric oxide (NO) bioavailability and are NO dependent (42).

Relaxin binds to a family of receptors classified as relaxin family peptide receptor (RXFP)1, RXFP2, RXFP3, and RXFP4. Relaxin can bind to both RXFP1 and RXFP2, but relaxin binds primary to RXFP1 in the kidneys (1, 27). RXFP1 receptors are in various arteries and veins and are differentially localized in endothelial and underlying VSM cells. The distribution of RXFP1 between these two cell types also differs among vessel type (25, 32, 34, 40, 55). Studies in rat aorta, vena cava, and mesenteric arteries and veins have shown that RXFP1 is expressed predominantly in endothelial cells. In contrast, RXFP1 is more highly expressed in VSM cells of uterine arteries, femoral arteries, and veins (25, 55). This differential pattern of RXFP1 localization in various artery types and veins lends support to the idea that serelaxin treatment will produce region-dependent effects on the vasculature, depending on the blood vessel type. This is best illustrated in animal studies that assessed vascular responses to exogenous serelaxin treatment in different arteries and veins. Both relaxin and RXFP1 are also expressed in small renal arteries of both male and female rats (34). Immunohistochemical analyses have further localized the expression of this receptor in the proximal tubules, inner medullary collecting ducts, and mesangial cells (3, 21). Serelaxin binds to a G-protein coupled RXFP1 receptor, which causes an increase in cAMP, leading to phosphatidylinositol 3-kinase and PKC, which may produce further cAMP production (27, 40). Serelaxin also activates the MAP kinase pathway and increases nitric oxide (NO) via activation of endothelial NO synthase (NOS) (27). Chronic relaxin administration decreases systemic vascular resistance and increases cardiac output and global arterial compliance. Relaxin also decreases renal vascular resistance, increases renal plasma flow, and inhibits myogenic constriction of isolated arteries (9, 30). Studies have shown that relaxin causes renal vasodilation with increases in renal plasma flow in both conscious and anesthetized rats (4, 12, 13). However, the effects on glomerular filtration rate (GFR) have been variable (4, 47). Clearance experiments in conscious rats have demonstrated that relaxin increases renal plasma flow, GFR, urine

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flow, and urinary excretion of sodium and chloride (11, 13). However, other studies in rats and human subjects did not show an increase in GFR (4, 47). The effects of relaxin on GFR are thus not clear and may be due to differential effects on the afferent and efferent arterioles, and although the presence of RXFP receptors on the endothelial cells and/or vascular smooth muscle cells of the afferent and efferent arterioles has not been established, RXFP1 has previously been localized to renal interlobar arteries (25, 34). It has also been suggested that endothelin acting on ET_B receptors as well as NO contributes to the renal vasodilation elicited by relaxin (12).

Our objectives of this study were to *1*) localize the specific immunoexpression of relaxin RXFP1 receptors in the normal rat kidney, *2*) determine the effects of serelaxin on overall renal function in normal rats and in rats with elevated angiotensin II (Ang II) levels to simulate the intrarenal environment present in heart failure and also because Ang II seems to have an antagonistic effect on the actions of NO in the kidney, 3) determine the effects of serelaxin on afferent and efferent arterioles in kidneys harvested from normal rats and under the conditions of NO inhibition, and *4*) determine the effects of serelaxin on afferent and efferent arterioles in the presence of increased Ang II concentrations.

METHODS

Renal function study. Experiments were performed on Sprague-Dawley male and female rats (275–350 g), using a protocol approved by the Tulane University Institutional Animal Care and Use Committee. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and placed on a surgical table thermostatically controlled to maintain body temperature at 37°C. A tracheostomy was performed, and the animals were allowed to breathe humidified air enriched with oxygen (95% O_2-5% CO_2). The left jugular vein was cannulated with a PE-50 catheter to allow infusion of solutions and additional anesthetic. The rats were infused with 0.9% saline containing 6% albumin (bovine; Sigma, St. Louis, MO) at a constant rate of 1.2 ml/h during surgery and thereafter with 0.9% saline containing 1% albumin and 7.5% polyfructosan (Inutest; Laevosan Geselschaft, Linz, Austria). The relatively low infusion rate of albumin containing isotonic solution has been utilized to maintain a euvolemic state and stable levels of arterial pressure, renal blood flow, urine flow, and Na excretion for ≤4 h (50). A PE-50 catheter was inserted into the left femoral artery to allow monitoring of arterial blood pressure and to facilitate collection of blood samples. Blood pressure was monitored with a Statham pressure transducer (model P23DC) and recorded using a computerized data acquisition system (MP100 System; Biopac Systems, Santa Barbara, CA) with the AcqKnowledge Software Package (version 3.7.3; Biopac). The left kidney was exposed via a subcostal flank incision and then freed from surrounding tissue and placed in a Lucite cup. A flow probe was placed on the renal artery to allow measurement of total renal blood flow. The left ureter was cannulated to allow timed urine collections to be obtained. After a 60-min recovery period, urine was collected during two 30-min control periods, followed by a control blood sample (~200 μl). Previous studies have infused serelaxin at a rate of 4 μg/h and produced plasma levels of 20–40 ng/ml, which are similar to the circulating relaxin concentrations measured in rats at 12–14 gestational days (5, 17, 45, 48, 56). Serelaxin was administered by intravenous bolus injection (5 μg in 40 μl of saline at 30 s; $n = 5$) or by infusion (2 μg·100 g body $wt^{-1}\cdot h^{-1}$; $n = 5$; or 5 μg·100 g body $wt^{-1}\cdot h^{-1}$; $n = 5$). Before infusion, a bolus injection was given (2 μg in 40 μl of saline in 30 s) to reach effective blood concentrations rapidly.

For experiments in rats having elevated Ang II levels, Ang II was infused at 3 ng/min for whole kidney experiments. After 60 min of Ang II infusion to establish steady-state effects of Ang II, serelaxin was administered by intravenous bolus injection of 2 μg in 40 μl of saline at 30 s, followed by infusion at 5 μg·100 g body $wt^{-1}\cdot h^{-1}$ ($n = 5$). After a 10-min equilibration period, urine was collected during six 30-min experimental periods, followed by collection of an additional blood sample (~200 μl). Mean arterial pressure (MAP), heart rate, renal blood flow, and urine flow were assessed during baseline conditions and following the Ang II and serelaxin experimental periods. Urine volume was determined gravimetrically. Sodium and potassium concentrations in urine were measured using flame photometry. Inulin concentrations in both urine and plasma samples were measured by standard spectrophotometry. GFR was estimated from the clearances of inulin (24).

Juxtamedullary nephron afferent and efferent arteriolar experiments. Afferent and efferent arteriolar diameters were assessed in vitro using the isolated blood-perfused juxtamedullary nephron technique combined with videomicroscopy, as previously described (20). Each experiment used one male Sprague-Dawley rat (Charles River Laboratories, Wilmington, MA) weighing 375–450 g and serving as blood donor and kidney donor. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and a cannula was inserted in the left carotid artery for blood collection. Donor blood was collected in a heparinized (500 U) syringe via the carotid arterial cannula and centrifuged to separate the plasma and cellular fractions. The buffy coat was removed and discarded. After sequential passage of the plasma through 5- and 0.22-μm filters (Gelman Sciences, Ann Arbor, MI), erythrocytes were added to achieve a hematocrit of 33%. This reconstituted blood was passed through a 5-μm nylon mesh and thereafter stirred continuously in a closed reservoir that was pressurized with a 95% O_2-5% CO_2 gas mixture.

The right kidney was perfused through a cannula inserted in the superior mesenteric artery and advanced into the right renal artery. The perfusate was a Tyrode's solution (pH 7.4) containing 5.1% BSA and a mixture of L-amino acids. The kidney was excised and sectioned longitudinally, retaining the papilla intact with the perfused dorsal two-thirds of the organ. The papilla was reflected to expose the pelvic mucosa and tissue covering the inner cortical surface. Overlying tissue was removed to expose the tubules, glomeruli, and related vasculature of the juxtamedullary nephrons. The arterial supply of the exposed microvasculature was isolated by ligating the larger branches of the renal artery.

After the dissection was completed, the Tyrode's perfusate was replaced with the reconstituted blood. Perfusion pressure was monitored by a pressure catheter centered in the tip of the perfusion cannula. Renal perfusion pressure was regulated by adjusting the rate of gas inflow into the blood reservoir and set at 100 mmHg. The inner cortical surface of the kidney was continuously superfused with a warmed (37°C) Tyrode's solution containing 1% BSA. The tissue was transilluminated on the fixed stage of a microscope (Nikon) equipped with a water immersion objective (×40). Video images of the microvessels were transferred by a Newvicon camera (model NC-67M; Dage-MTI, Michigan City, IN) through an image enhancer (model MFJ-1452; MFJ Enterprises, Starkville, MS) to a video monitor (Conrac Display Systems, Covina, CA). The video signal was recorded on DVD for later analysis. Afferent and efferent arteriolar inside diameters were measured at 5-min intervals using a calibrated digital image-shearing monitor (Instrumentation for Physiology and Medicine, San Diego, CA). Single afferent or efferent arterioles were visualized. Treatments were administered by superfusing the tissue with a Tyrode's solution containing the agent to be tested or vehicle. Ang II (0.5 nM) or N^G-nitro-L-arginine (L-NNA; 100 μM) was superfused with Tyrode's solution. Serelaxin (20 and 40 ng/ml) was infused by injecting it into the blood chamber. A single afferent or efferent arteriole that showed adequate blood flow was selected for study. After a 10-min equilibration period, an experimental protocol was initiated, consisting of consecutive 25-min treatment periods.

These doses were selected on the basis of pilot experiments using various doses.

The first experimental protocol was performed to determine the effects of serelaxin on afferent ($n = 5$) and efferent ($n = 5$) arterioles from normal rat kidneys. After a 20-min control period, serelaxin (20 ng/ml) was added to blood and infused for 25 min. It was then changed to 40 ng/ml serelaxin for another 25 min.

The second experimental protocol was performed to determine the effects of serelaxin on vasodilation of afferent ($n = 5$) and efferent ($n = 5$) arterioles in the presence of increased Ang II concentrations. After a 10-min control period, Ang II (0.5nM) was superfused for duration of the experimental period. At 20 min of Ang II superfusion, serelaxin (20 ng/ml) was infused in blood for 25 min and then changed to 40 ng/ml serelaxin for another 25 min.

The third experimental protocol was performed to determine the effects of serelaxin on afferent arterioles ($n = 5$) in the presence of nitric oxide synthase inhibitor L-NNA. After a 10-min control period, L-NNA (100 μM) was superfused for the duration of the experimental period. At 20 min of L-NNA superfusion, serelaxin (20 ng/ml) was infused in blood for 40 min.

Specific immunoexpression of the relaxin family peptide receptors in the normal rat kidney. To examine the specific immunoreactivity of relaxin receptors in the rat kidney, 3-μm paraffin-embedded sections were subjected to peroxidase technique and tyramide signal amplification using rabbit anti-RXFP1 (1 μg/ml) antibody (Cct. no. HPA027067; Sigma, St. Louis, MO) incubated overnight at 4°C. According to the manufacturer, antibodies are validated and approved for immunohistochemistry. To further validate the RXFP1 antibody, we performed preabsorption in consecutive sections preincubated with ×30 in excess of recombinant RXFP1 peptide for 72 h at 4°C. The specific immunostaining signal (brown, diaminobenzidine; Fig. 1*A*) of RXFP1 decreased substantially in glomerular cells, collecting ducts and renal arterioles in the section with preabsorption (Fig. 1*B*). To assist in cell type-specific localization, various additional known immunomarkers were used at room temperature for 90 min to c-localize the corresponding RXFP receptors. For vascular smooth muscle cells: anti-smooth muscle actin antibody (Dako Agilent Pathology Solutions, Santa Clara, CA) was utilized at 1:100 dilution; for endothelial cells, anti- PECAM1 (anti-CD31) antibody (Abcam, Cambridge, MA) was utilized at 1:500 dilution, and for principal cells, anti-aquaporin 2 antibody (Santa Cruz Biotechnology, Dallas, TX) was utilized at 1:800 dilution. The secondary antibodies Alexa Fluor donkey anti-mouse or donkey angi-rabbit, both in red, were used at 1:1,000 dilution RT (Invitrogen, Molecular Probe, Eugene, OR) for 1 h. Slow Fade DAPI reagent (Invitrogen; Molecular Probes) was used for the visualization of nucleus (blue). Visualization of results and capturing of images was done on a Nikon Eclipse 50i microscope using NIS Elements BR software.

Statistical analysis. Results are expressed as means ± SE. Data were analyzed by repeated-measures ANOVA with post hoc Newman-Keuls multiple-comparison test within each group and by one-way ANOVA with post hoc Newman-Keuls multiple-comparison test between groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Specific immunoexpression of the relaxin family peptide receptors in the normal rat kidney. RXFP1 was expressed inside glomerulus and on vascular smooth muscle cells, where it colocalized with α-smooth muscle actin of the renal vasculature and afferent arteriole (Fig. 2, *A* and *B*). It was also expressed on macula densa cells (Fig. 2*C*) and on endothelial cells based on colocalization with CD31+ in interlobar arterioles (Fig. 2, *D–F*). It also colocalized with AQP2 in principal cells of the collecting ducts (Fig. 2, *G* and *H*).

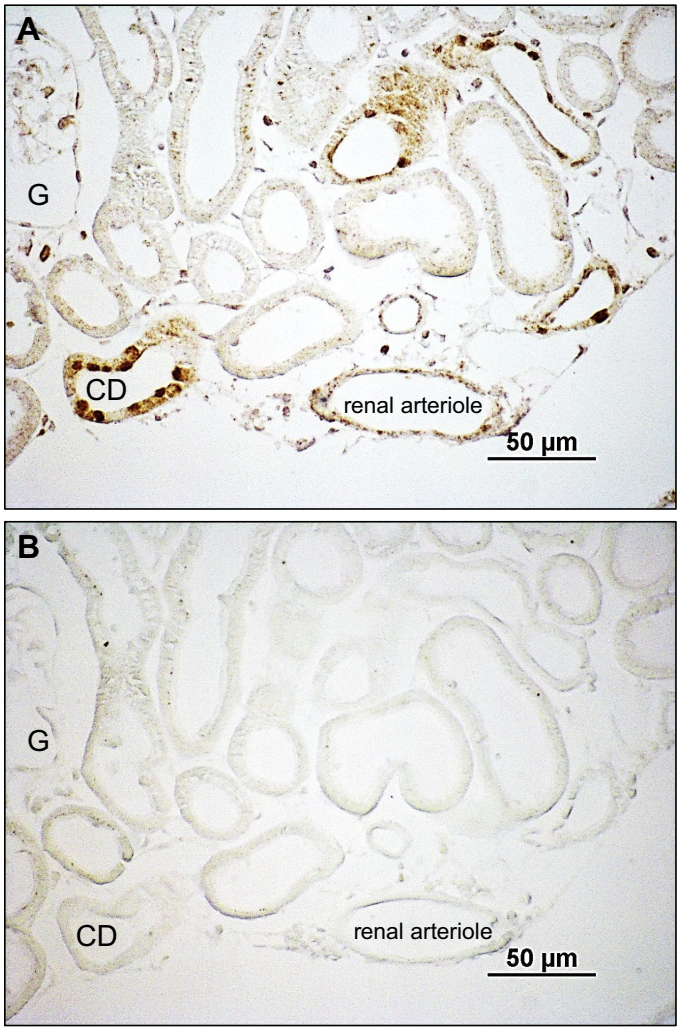

Fig. 1. *A*: relaxin family peptide receptor 1 (RXFP1) immunostaining [brown, diaminobenzidine (DAB) chromogen] was detected in glomerulus (G), collecting duct (CD), and vascular smooth muscle cells of a renal arteriole in male rat kidney section (3 μm). *B*: specificity of the rabbit anti-RXFP1 antibody was validated by preabsorption using ×30 in excess RXFP1 peptide. A substantial decrease of the specific RXFP1 immunostaining was observed in the true consecutive male rat kidney section.

Renal function study. In the group given serelaxin as a 5-μg bolus injection (Table 1), the MAP was slightly increased during the experimental period and was statistically different at 90,120, and 240 min compared with the control period (116 ± 1.5 vs. 123 ± 3.4 mmHg, $P < 0.05$; Table 1). Serelaxin significantly increased heart rate during the entire experimental period (386 ± 13 vs. 511 ± 20 beats/min, $P < 0.05$). Renal blood flow was consistently elevated after the bolus injection (7.2 ± 0.7 vs. 8.6 ± 0.9 $ml \cdot min^{-1} \cdot g^{-1}$, $P < 0.05$). Serelaxin significantly decreased renal vascular resistance in control rats as compared with the control period (16.6 ± 1.4 vs. 14.8 ± 1.5 $mmHg \cdot ml^{-1} \cdot min^{-1} \cdot g^{-1}$, $P < 0.05$). Urine flow was increased during 90- to 120-min periods and then gradually returned to the control period levels (5.2 ± 0.4 vs. 9.2 ± 1.5 $\mu l \cdot min^{-1} \cdot g^{-1}$, $P < 0.05$). GFR was significantly increased at the 240-min period compared with the control period (0.72 ± 0.07 vs. 0.99 ± 0.18 $ml \cdot min^{-1} \cdot g^{-1}$, $P < 0.05$). Serelaxin markedly enhanced sodium excretion and fractional

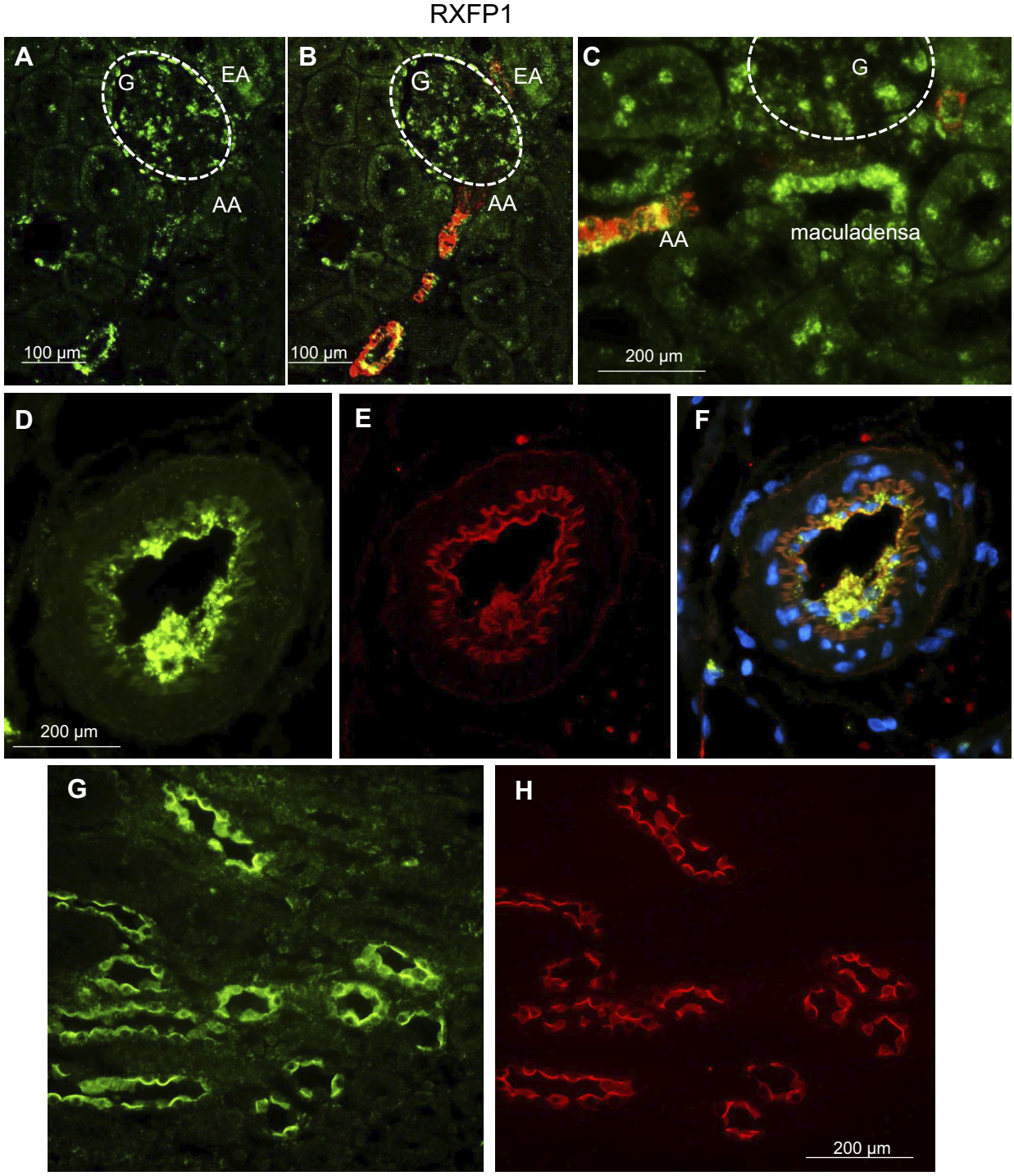

Fig. 2. *A*: RXFP1 (green) positive staining in the glomerulus. *B*: merged imaging showing colocalization of RXFP1 (green) and smooth muscle actin (SMA; red) in the afferent and efferent arterioles. *C*: RXFP1-positive staining (green) merged with SMA (red) in an afferent arteriole. Macula densa cells also express RXPF1 (green). *D–F*: colocalization of RXFP1 (green; *D*) and CD31 (PECAM-1, red; *E*) in a renal interlobar arteriole segment (merged images; *F*) denoting the presence of RXFP1 in endothelial cells. *G* and *H*: colocalization of RXFP1 (green; *G*) and aquaporin 2 (red; *H*) in principal cells of collecting ducts in true consecutive sections (3 μm). G, glomerulus; AA, afferent arteriole; EA, efferent arteriole. Scale is noted on each image.

sodium excretion rates, with a slight waning effect during the later period (0.2 ± 0.05 vs. 1.4 ± 0.3 $\mu mol \cdot min^{-1} \cdot g^{-1}$, $P < 0.05$). The potassium excretion rate was increased by the 90-min period (2.1 ± 0.1 vs. 2.5 ± 0.2 $\mu mol \cdot min^{-1} \cdot g^{-1}$, $P < 0.05$) but then gradually decreased to levels lower than in the control period (2.1 ± 0.1 vs. 1.7 ± 0.1 $\mu mol \cdot min^{-1} \cdot g^{-1}$, $P < 0.05$).

In the serelaxin infusion group, at an infusion of 2 $\mu g \cdot 100$ g body $wt^{-1} \cdot h^{-1}$, MAP was significantly elevated at the 240-min collection period (Table 2). Serelaxin significantly increased

Table 1. *Effects of serelaxin (5-μg bolus injection) on renal function in normotensive male rats*

	Control Period	After Injection
MAP, mmHg	116 ± 1.5	123 ± 3.4*
Heart beat, beats/min	386 ± 13	511 ± 20*
RBF, ml·min^{-1}·g^{-1}	7.2 ± 0.7	8.6 ± 0.9*
RVR, mmHg·ml^{-1}·min^{-1}·g^{-1}	16.6 ± 1.4	14.8 ± 1.5*
UF, μl·min^{-1}·g^{-1}	5.2 ± 0.4	9.2 ± 1.5*
GFR, ml·min^{-1}·g^{-1}	0.72 ± 0.07	0.99 ± 0.18*
Na excretion, μmol·min^{-1}·g^{-1}	0.2 ± 0.05	1.4 ± 0.3*
FENa, %	0.17 ± 0.03	1.13 ± 0.24*
K excretion, μmol·min^{-1}·g^{-1}	2.1 ± 0.1	2.5 ± 0.2 and 1.7 ± 0.1*

Values are means ± SE. MAP, mean arterial pressure; RBF, renal blood flow; RVR, renal vascular resistance; UF, urine flow; GFR, glomerular filtration rate; FENa, fractional Na excretion. *$P < 0.05$ vs. control period; $n = 5$.

heart rate during the entire experimental period. Renal blood flow was significantly increased from the 90-min period to the 240-min period. Serelaxin significantly decreased renal vascular resistance in control rats compared with the control period. Urine flow was not significantly different between the control period and experimental period. In this group, there were no significant differences in GFR between control periods and experimental period. Sodium excretion and fractional sodium excretion rates were increased starting at the 120-min period compared with the control period. Serelaxin did not change the potassium excretion rate.

In the serelaxin infusion group at 5 μg·100 g body wt^{-1}·h^{-1}, MAP significantly increased by the 90-min collection period and remained elevated until the 240-min period compared with control measurements (118 ± 3.3 vs.132 ± 3.7 mmHg, $P < 0.05$; Fig. 3*A*). In Ang II-infused rats, serelaxin did not alter MAP (121 ± 2.5 vs. 125 ± 1.9 mmHg; Fig. 3*A*). Heart rate remained increased for the duration of the experiment in the serelaxin infusion group (373 ± 16.5 vs. 508 ± 8.3 beats/min, $P < 0.05$; Fig. 3*B*) and in Ang II-infused rats (453 ± 18.5 vs. 515 ± 12.7 beat/min, $P < 0.05$; Fig. 3*B*). Renal blood flow was significantly increased starting at the 120-min to 240-min period compared with the control period (average: 7.4 ± 0.6 vs. 9.4 ± 0.5 ml·min^{-1}·g^{-1}, $P < 0.05$; Fig. 4*A*). In Ang II-infused rats, serelaxin did not alter renal blood flow (average: 7.5 ± 0.4 vs. 7.9 ± 0.5 ml·min^{-1}·g^{-1}, Fig. 4*A*). Serelaxin did not significantly decrease renal vascular resistance, but the lower value during serelaxin infusion suggested a slight vasodilation (16 ± 1.2 vs. 14 ± 0.8 mmHg·ml^{-1}·min^{-1}·g^{-1}; Fig. 4*B*). However, renal vascular resistance was not altered compared with the Ang II infusion-only period (16.3 ± 0.8 vs. 16 ± 0.8 mmHg·ml^{-1}·min^{-1}·g^{-1}; Fig. 4*B*). In this series, GFR was not significantly increased during the 90- and 240-min periods compared with the 60-min period (1.1 ± 0.1 vs. 1.3 ± 0.1 ml·min^{-1}·g^{-1}; Fig. 5*A*) in control rats. Serelaxin also did not significantly alter GFR between the 90- to 120-min and 150- to 240-min period in Ang II-infused rats (1.3 ± 0.3 vs. 1.5 ± 0.2 ml·min^{-1}·g^{-1}; Fig. 5*A*). Urine flow was significantly increased starting at the 120-min period and remained elevated until the 240-min period compared with the control period (5.0 ± 0.4 vs. 11.1 ± 2.2 μl·min^{-1}·g^{-1}, $P < 0.05$ Fig. 5*B*). However, serelaxin did not alter urine flow in the Ang II-infused rats (4.9 ± 0.5 vs. 4.9 ± 0.5 μl·min^{-1}·g^{-1}; Fig. 5*B*). Serelaxin increased sodium excretion rate at the 120-min period compared with control period, with peak sodium excretion at the 180-min period, which remained elevated for the duration of the experiment (0.14 ± 0.03 vs. 2.05 ± 0.36 μmol·min^{-1}·g^{-1}, $P < 0.05$; Fig. 6*A*). However, in the Ang II-infused rats, sodium excretion rate was statistically elevated only after 210-min period (0.44 ± 0.15 vs. 1.16 ± 0.35 μmol·min^{-1}·g^{-1}, $P < 0.05$; Fig. 6*A*). Serelaxin significantly increased fractional Na excretion (FENa) at the 120-min period

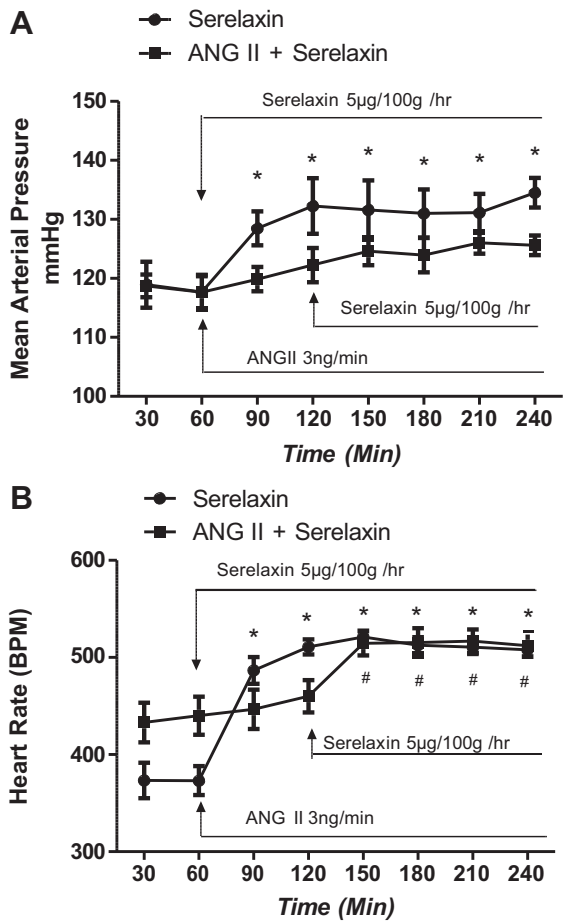

Fig. 3. *A*: effects of serelaxin infusion on mean arterial pressure in control ($n = 5$) and angiotensin II (Ang II)-infused male rats ($n = 5$). *B*: effects of serelaxin infusion on heart rate in control ($n = 5$) and Ang II-infused male rats ($n = 5$). Values are means ± SE. In both *A* and *B*, a period of serelaxin infusion alone is designated (*top*), and a period of Ang II infusion followed by serelaxin infusion is indicated (*bottom*). *$P < 0.05$ vs. 60-min period; #$P < 0.05$ vs. 120-min period. BPM, beats/min.

Table 2. *Effects of serelaxin (2 μg·100 g body wt^{-1}·h infusion^{-1}) on renal function in normotensive male rats*

	Control Period	After Injection
MAP, mmHg	123 ± 3.5	133 ± 3.5*
Heart beat, beats/min	395 ± 21	524 ± 16*
RBF, ml·min^{-1}·g^{-1}	7.3 ± 0.8	9.3 ± 0.7*
RVR, mmHg·ml^{-1}·min^{-1}·g^{-1}	17.6 ± 1.8	14.3 ± 0.9*
UF, μl·min^{-1}·g^{-1}	4.5 ± 0.3	6.3 ± 0.7
GFR, ml·min^{-1}·g^{-1}	1.0 ± 0.1	1.1 ± 0.1
Na excretion, μmol·min^{-1}·g^{-1}	0.2 ± 0.06	1.0 ± 0.2*
FENa, %	0.08 ± 0.01	0.77 ± 0.17*
K excretion, μmol·min^{-1}·g^{-1}	1.7 ± 0.3	1.9 ± 0.3

Values are means ± SE. *$P < 0.05$ vs. control period; $n = 5$.

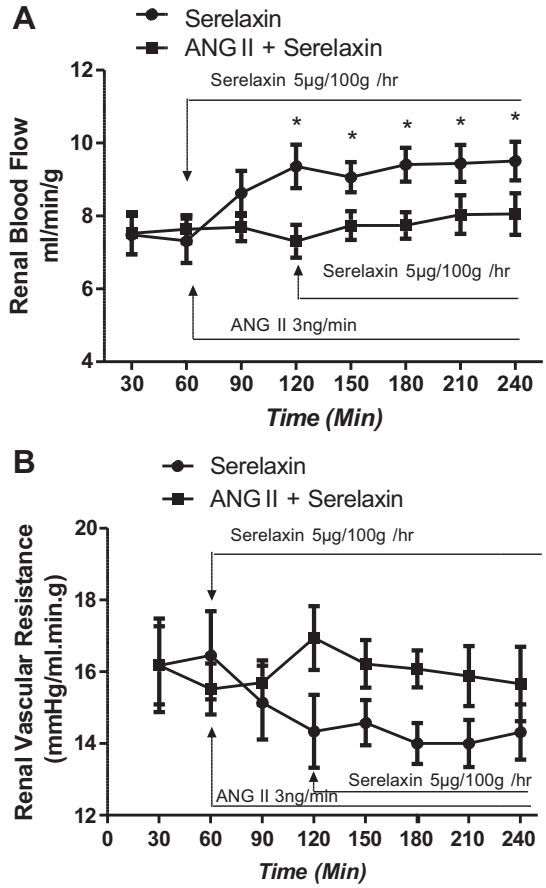

Fig. 4. *A*: effects of serelaxin infusion on renal blood flow in control ($n = 5$) and Ang II-infused male rats ($n = 5$). *B*: effects of serelaxin infusion on renal vascular resistance in control ($n = 5$) and Ang II-infused male rats ($n = 5$). Values are means ± SE. Designations are the same as in Fig. 3. *$P < 0.05$ vs. 60-min period.

compared with the control period (0.08 ± 0.02 vs. 1.04 ± 0.15%; Fig. 6*B*). In the Ang II-infused rats, serelaxin did not elevate FENa compared with the Ang II infusion-only period (0.35 ± 0.18 vs. 0.46 ± 0.17%; Fig. 6*B*). Potassium excretion rate was elevated only at the 90- and 120-min periods compared with the control period and then returned to values not significantly different from control values (1.67 ± 0.22 vs. 2.49 ± 0.17 μmol·min^{-1}·g^{-1}, $P < 0.05$). Serelaxin did not change potassium excretion rate in Ang II-infused rats (1.78 ± 0.18 vs. 1.78 ± 0.11 μmol·min^{-1}·g^{-1}). Because GFR was not significantly increased in this group, we performed a group comparison of peak GFR values compared with the control period values, including all experiments in which serelaxin was administered either as a 5-μg bolus injection or as an infusion, including the 2 and 5 μg·100 g^{-1}·h^{-1} ($n = 15$). In agreement with data shown in Table 1, we were able to confirm that GFR increased significantly during serelaxin infusion (60-min control period: 0.93 ± 0.06 vs. 210- to 240-min period: 1.16 ± 0.08 ml·min^{-1}·g^{-1}, $P < 0.05$).

In female rats, serelaxin at 5 μg·100 g body wt^{-1}·h^{-1} infusion increased the MAP significantly from the 90-min collection period and remained elevated until the 240-min period compared with control measurements (Table 3). Serelaxin significantly increased heart rate during the entire experimental period. Renal blood flow was significantly increased starting at the 150-min to 240-min period compared with the control period. Urine flow was significantly increased from the 180-min to 210-min period compared with control period. There was no significant difference in GFR between the control period and experimental periods in this group. Sodium excretion and fractional sodium excretion rates were elevated at the 180-min period compared with the control period and remained elevated for the duration of the experiment (0.28 ± 0.08 vs. 2.14 ± 0.49 μmol·min^{-1}·g^{-1}, $P < 0.05$). Potassium excretion rate was significantly elevated from the 150- to 240-min period compared with the control period (1.06 ± 0.16 vs. 2.27 ± 0.45 μmol·min^{-1}·g^{-1}, $P < 0.05$).

Juxtamedullary nephron afferent and efferent arteriolar study. In control kidneys, serelaxin at 20 and 40 ng/ml infusion in blood significantly dilated afferent arterioles (10.8 ± 1.2 vs. 12.7 ± 1.1 and 13.5 ± 1.1 μm, $P < 0.05$; Fig. 7). Interestingly, in the rats with Ang II superfusion (0.5 nM), serelaxin at 20 ng/ml in blood had no effects on afferent arteriolar diameters (9.4 ± 0.2 vs. 9.5 ± 0.2 μm), but serelaxin infused at 40 ng/ml in blood exerted slight but statistically significant afferent arteriolar vasodilation compared with the 20- to 25-min period (9.3 ± 0.2 vs. 9.7 ± 0.2 μm, $P < 0.05$; Fig. 7). In control rat kidneys, serelaxin at 20 and 40 ng/ml infusion in

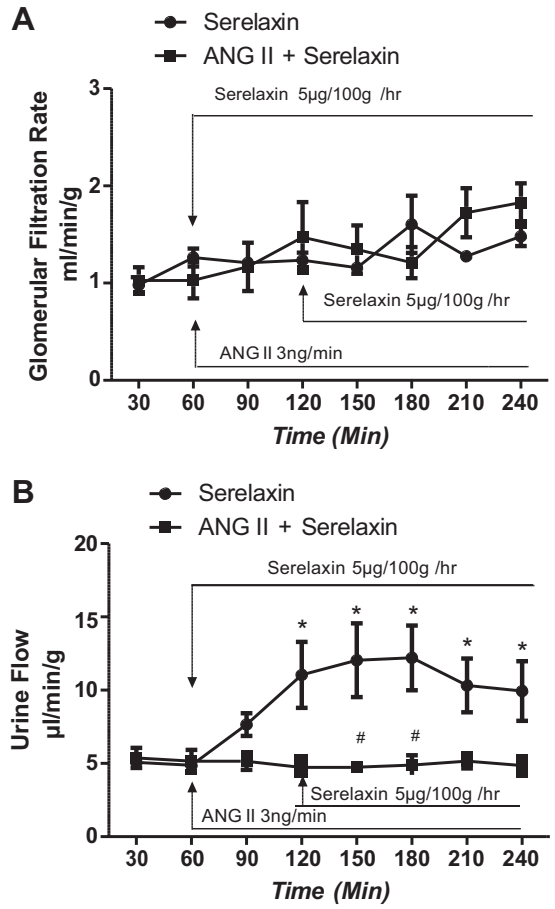

Fig. 5. *A*: effects of serelaxin infusion on glomerular filtration rate in control ($n = 5$) and Ang II-infused male rats ($n = 5$). *B*: effects of serelaxin infusion on urine flow in control ($n = 5$) and Ang II-infused male rats ($n = 5$). Values are means ± SE. Designations are the same as in Fig. 3. *$P < 0.05$ vs. 60-min period; #$P < 0.05$ vs. serelaxin only.

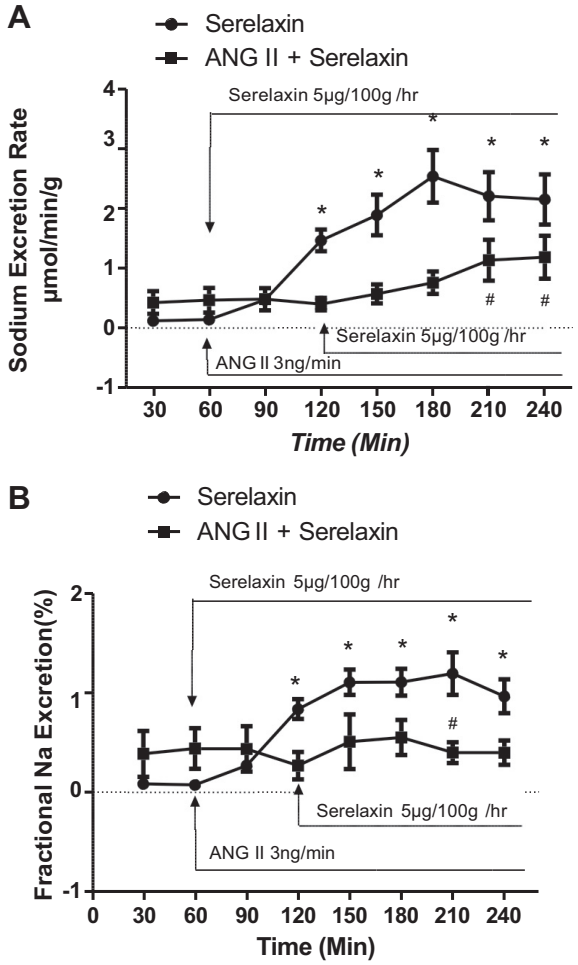

Fig. 6. *A*: effects of serelaxin infusion on sodium excretion rate in control (n = 5) and Ang II-infused male rats (n = 5). *B*: effects of serelaxin infusion on fractional Na excretion in control (n = 5) and Ang II-infused male rats (n = 5). Values are means ± SE. Designations are the same as in Fig. 3. *$P < 0.05$ vs. 60-min period; #$P < 0.05$ vs. 120-min period (in *A*) and vs. serelaxin infusion only (in *B*).

blood significantly dilated efferent arterioles (9.9 ± 0.9 vs. 11.2 ± 1.1 and 11.9 ± 1.0 μm, $P < 0.05$; Fig. 8). However, serelaxin at either 20 or 40 ng/ml infusion in blood did not alter the efferent arteriolar diameters in Ang II-superfused rat kidneys (9.1 ± 0.2 vs. 9.0 ± 0.2 and 9.1 ± 0.2 μm; Fig. 8). As previously shown, superfusion with L-NNA elicited substantial afferent arteriolar constriction (Fig. 9). In the L-NNA-superfused rat kidneys, serelaxin failed to alter the afferent arteriolar diameters (9.5 ± 0.4 vs. 9.4 ± 0.5 μm; Fig. 9).

Table 3. *Effects of serelaxin (5 μg·100 g body wt^{-1}·h infusion^{-1}) on renal function in normotensive female rats*

	Control Period	After Injection
MAP, mmHg	119 ± 3.9	129 ± 2.4*
Heart beat, beats/min	443 ± 19.2	532 ± 10.9*
RBF, ml·min^{-1}·g^{-1}	7.6 ± 0.5	10.8 ± 1.1*
RVR, mmHg·ml^{-1}·min^{-1}·g^{-1}	16.0 ± 1.7	13.0 ± 1.1
UF, μl·min^{-1}·g^{-1}	6.5 ± 1.4	12.1 ± 2.6*
GFR, ml·min^{-1}·g^{-1}	0.83 ± 0.09	0.97 ± 0.11
Na excretion, μmol·min^{-1}·g^{-1}	0.28 ± 0.08	2.14 ± 0.49*
FENa, %	0.27 ± 0.09	1.51 ± 0.4*
K excretion, μmol·min^{-1}·g^{-1}	1.06 ± 0.16	2.27 ± 0.45*

Values are means ± SE. *$P < 0.05$ vs. control period; n = 5.

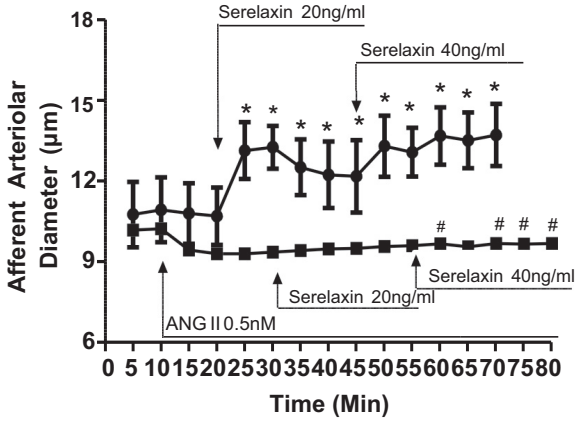

Fig. 7. Effects of serelaxin infusion on afferent arteriolar diameters in control (n = 5) and Ang II-superfused male rat kidneys (n = 5). Values are means ± SE. *$P < 0.05$ vs. 20-min period; #$P < 0.05$ vs. 20, 25 min.

DISCUSSION

Relaxin plays a key role in the maternal hemodynamic and renal adjustments that occur during pregnancy (17, 33, 44, 48, 53). Relaxin-2 binds primarily to relaxin family peptide receptor 1 (RXFP1) located in the heart, kidneys, and vasculature to activate numerous cellular pathways (18, 23, 40, 52). Serelaxin has been manufactured as the recombinant human relaxin-2 and has been evaluated for treatment of acute heart failure (2, 51–53). It is known that serelaxin stimulates vasorelaxation systems and counteracts vasoconstrictor systems and mediates both rapid and sustained vasorelaxation to improve hemodynamics and alleviate congestion (9, 19, 28). Previous reports indicate that serelaxin increases cardiac output and systemic arterial compliance and reduces systemic vascular resistance irrespective of sex (10, 14–16). In our current study, acute infusion of serelaxin slightly elevated MAP in normal anesthetized male and female rats. Heart rate was also significantly increased by serelaxin infusion in both male and female rats. The increase of MAP varied from 6 to 12%, and the increase in heart rate was from 20 to 37% at different doses of serelaxin

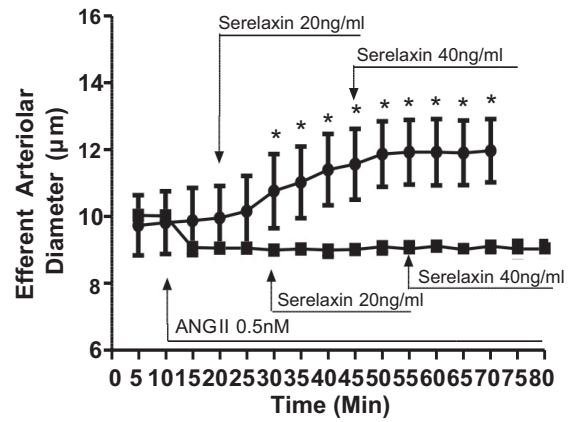

Fig. 8. Effects of serelaxin infusion on efferent arteriolar diameters in control (n = 5) and Ang II-superfused male rat kidneys (n = 5). Values are means ± SE. *$P < 0.05$ vs. 20-min period.

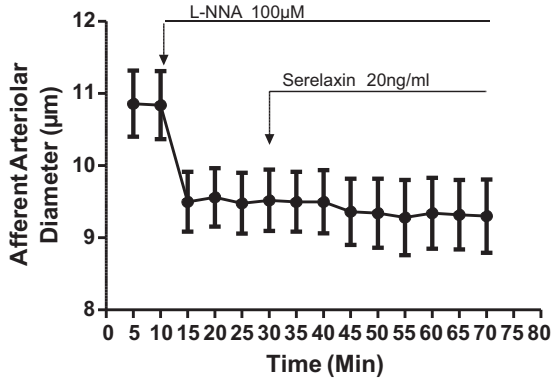

Fig. 9. Effects of serelaxin infusion on afferent arteriolar diameter in N^G-nitro-L-arginine (L-NNA)-superfused male rat kidneys (n = 5). Values are means ± SE.

administration. Debrah et al. (14) reported small (8%) increases in MAP and 13% increases in heart rate in response to short-term serelaxin administration in female rats. The increases of heart rate and MAP started at 90 min, and systemic hemodynamic changes started at 120 min. The mild increases of MAP may be due to the increases of heart rate, which presumably increased cardiac output. However, other mechanisms may have also contributed. In another study, chronic infusion of serelaxin for 3 wk slightly reduced mean arterial pressure in normotensive rats (42). Relaxin also reduced blood pressure in Ang II-induced hypertensive rats but had no protective effect on blood pressure during hypertension induced by chronic NOS inhibition (42).

Chronic infusion of relaxin in conscious rats induced renal vasodilation and increased GFR comparable with that observed during pregnancy (8, 12, 13). Relaxin produced renal vasodilation in intact and ovariectomized female rats (13) and in male rats (12). By using relaxin (RLX)-neutralizing antibodies or removing circulating RLX by ovariectomy, the gestational renal vasodilation and reduced myogenic reactivity of small renal arteries isolated from midterm pregnant rats were abolished (33). In our studies, acute administration of serelaxin significantly elevated renal blood flow in normal male and female rats, but arterial pressure also increased such that the renal vascular resistance remained unchanged or decreased only slightly. However, with autoregulation intact, the renal vascular resistance would have increased, suggesting that the serelaxin kept the renal vascular resistance from exhibiting an autoregulatory-induced increase in renal vascular resistance. In previous reports, acute serelaxin infusion induced significant increases in renal blood flow within 45 min of the start of infusion (4) and 1–2 h after the intravenous infusion (11), which is similar to our results.

Although GFR was increased significantly in rats given the 5-μg bolus, the GFR was not consistently increased during short periods of serelaxin infusion in male and female rats. It was also reported that short-term infusion of relaxin did not have effects on GFR in anesthetized rats (4, 13). Recognizing the limitations of the clearance technique that make it difficult to establish significances when there are small changes in GFR, we grouped all studies with serelaxin at 2 and 5 μg, which allowed us to establish a significant increase in GFR. The lack of a consistent effect of serelaxin on GFR can be explained by our results from the juxtamedullary nephron studies, which showed similar effects of serelaxin to dilate both afferent and efferent arterioles. Compared with previous studies of short-term infusion of relaxin, which increased GFR in conscious rats (11–13), the present study had lower baseline of renal blood flow and GFR in our anesthetized rats. Therefore, anesthesia and acute surgical stress may be one of the reasons for the smaller effects on GFR. Nevertheless, several regulatory mechanisms control renal blood flow and GFR through differential control of the vascular tone of the afferent and efferent arterioles, which allows the kidneys to maintain GFR and renal blood flow under various physiological conditions. Serelaxin dilated both the afferent and efferent arterioles to similar extents, so the glomerular hydrostatic pressure may not have increased, but the increase in plasma flow could increase GFR slightly. Collectively, our studies support small increases in GFR, in agreement with some previous reports (11, 12). The increases in GFR could have contributed to the increases in urine flow and sodium excretion, but direct effects of serelaxin to decrease sodium reabsorption are also likely.

Short-term infusion of serelaxin significantly increased urine flow and sodium excretion rate in both male and female rats. Studies have shown that relaxin stimulates drinking in both pregnant (36) and nonpregnant rats (46). Short-term serelaxin infusion had no effect on urine flow but increased the sodium excretion, and long-term serelaxin infusion for 7 days increased both urine flow and sodium excretion and decreased plasma osmolality and plasma sodium concentration (4).

In our RXFP1 immunoexpression studies, the RXFP1 receptor was localized on principal cells of the collecting ducts as well as on the vascular cells. Other studies have also found that RXFP1 is expressed on the apical membrane of the tubular cells of the renal cortex in the pregnant and nonpregnant rats and also in the glomeruli of pregnant rats (21), but it was not apparent that the antibodies used were validated. The presence of the RXPF1 receptors on the luminal membrane of principal cells suggests an interaction of serelaxin with tubular receptors that may inhibit tubular sodium reabsorption, leading to increases in sodium excretion even in the absence of changes in GFR. In addition, relaxin activates the ET_B receptor (12), which is expressed in the thick ascending limb of the loop of Henle (37), the cortical collecting duct (54), and the inner medullary collecting duct (26) and could contribute to the natriuresis (57). The increases in sodium excretion and urine flow, coupled with the small changes in GFR, suggest that serelxain induced a natriuresis by inhibiting tubular reabsorption of sodium. In addition, relaxin stimulates NO and ultimately cGMP production, the latter reducing the open probability time of the amiloride-sensitive apical sodium channel (29). From these data, it is not possible to determine the specific sodium transporters that are inhibited, but the localization studies suggest an inhibition of the amiloride-sensitive Na channels in the principal cells.

Because patients with heart failure have a stimulation of the renin-angiotensin system, we also examined the effects of serelaxin in rats infused with a subthreshold Ang II dose that did not affect renal perfusion pressure or renal blood flow. In the presence of Ang II, serelaxin did not alter MAP, renal blood flow, GFR, urine flow, sodium excretion, or potassium excretion compared with the responses to serelaxin alone. Although serelaxin still increased heart rate during subthreshold Ang II infusion, the

increase in heart rate was less. Other studies also suggest that administration of relaxin opposes the action of Ang II in vitro and vivo (13, 15, 39). The mechanisms by which Ang II blunts the hemodynamic and renal functional responses to serelaxin may be due to Ang II-mediated actions on endogenous NOS inhibitor, asymmetric dimethylarginine, or superoxide-induced increases in oxidative stress that eventually affect NO bioavailability (39, 41). In Ang II-infused conscious rats, chronic administration of serelaxin reduced MAP (42), increased cardiac output, and decreased systemic vascular resistance (15). Relaxin also reduced MAP, albumin excretion, and oxidative stress markers in Ang II-infused hypertension rats but did not attenuate these changes in rats made hypertensive by treatment with NOS inhibitors (42). Also, chronic administration of relaxin blunted renal vasoconstrictor responses to an infusion of angiotensin II in conscious rats (13). The apparent discrepancies between the present and previous studies are likely due to differences in experimental design such as conscious, chronically instrumented vs. anesthetized, acutely prepared animals, chronic vs. acute infusions of relaxin, and order of angiotensin II and relaxin administration.

In the studies utilizing the juxtamedullary nephron preparation, serelaxin significantly dilated afferent and efferent arterioles in control conditions. However, in Ang II-superfused rat kidneys, the afferent and efferent arteriolar vasodilatory responses to serelaxin were markedly attenuated and did not show significant vasodilation, except for a small effect on afferent arterioles at 40 ng/ml. In preparations treated with an NOS inhibitor, afferent arterioles failed to show any vasodilation in response to serelaxin infusion. These results are consistent with in vivo studies showing that the antihypertensive and renal vasodilator and protective effects of relaxin are dependent on a functional NOS system (13, 42). Collectively, our results demonstrate that serelaxin has dilating actions on both pre- and postglomerular arterioles, which are either attenuated or prevented in the presence of Ang II. This inhibition also occurred in the presence of nitric oxide synthase inhibition, which prevented the serelaxin-induced vasodilation. In other studies, the myogenic reactivity of renal interlober arteries from pregnant rats and relaxin-treated rats was reduced, but treatment of arteries with a NO synthesis inhibitor or ET_B receptor antagonist or endothelial removal restored the reduced myogenic reactivity to the levels in renal interlober arteries from virgin rats (22, 35).

The molecular mechanisms by which relaxin activates NOS and induces renal vasodilation remain unclear. Conrad (9) has implicated the two-phase mechanisms for the renal vasodilatory response to relaxin: *1*) the rapid vasodilatory responses of relaxin by binding to RXFP1 receptor, which induces Gαi/o protein coupling to phosphatidylinositol 3-kinase/Akt (PKB)-dependent phosphorylation and activation of endothelial NOS and *2*) the sustained vasodilatory responses due to the activation of vascular gelatinases, which result in stimulation of NO vasodilatory pathway. Further investigations are still needed to elucidate the molecular mechanisms of these vasodilatory effects.

In conclusion, serelaxin increases overall renal blood flow, urine flow, and sodium excretion with smaller and variable increases in GFR that improve renal function and the renal microcirculation via vasodilation of both renal pre- and postglomerular arterioles in control conditions. These effects are reduced or prevented in the presence of exogenous Ang II and NOS inhibitor, suggesting that some of the actions are mediated through activation of receptors on endothelial cells and release of NO. Additional in vivo effects indicate that serelaxin also causes natriuresis, which may be due to direct effects to inhibit tubular reabsorption as well as small increases in GFR. Overall, no deleterious effects of serelaxin on kidney function or the renal microcirculation were detected.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

W.S., M.C.P., and L.G.N. conceived and designed research; W.S., C.B.R., and C.G. performed experiments; W.S. and C.B.R. analyzed data; W.S., C.B.R., M.C.P., and L.G.N. interpreted results of experiments; W.S., C.B.R., and M.C.P. prepared figures; W.S., C.B.R., and M.C.P. drafted manuscript; W.S. and L.G.N. edited and revised manuscript; W.S. and L.G.N. approved final version of manuscript.

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