

Acute Intravenous Injection of Serelaxin (Recombinant Human Relaxin-2) Causes Rapid and Sustained Bradykinin-Mediated Vasorelaxation

Chen Huei Leo, PhD; Maria Jelinic, MSc; Helena C. Parkington, PhD; Marianne Tare, PhD; Laura J. Parry, PhD

Background—A recent clinical trial (RELAXin in Acute Heart Failure [RELAX-AHF]) demonstrated that 48 hours of continuous intravenous infusion of the vasorelaxant peptide serelaxin (recombinant human relaxin-2) to patients with acute heart failure reduced cardiovascular mortality at 180 days. The persistence of a vasorelaxant response as a potential mechanism for this long-term benefit and the vascular effects of a bolus intravenous injection of serelaxin have not been examined. This study investigates changes in resistance artery reactivity and passive mechanical wall properties following an intravenous serelaxin injection and whether these vascular effects persist in the absence of detectable circulating serelaxin.

Methods and Results—Male rats were injected with 13.3 µg/kg serelaxin into the tail vein; mesenteric arteries were assessed 3 and 24 hours after treatment by using wire-myography. Serelaxin increased basal nitric oxide synthase activity and reduced maximal contraction to endothelin-1 at 3 hours after administration. Serelaxin treatment also selectively enhanced bradykinin-mediated endothelium-dependent relaxation. This effect was sustained for 24 hours in the absence of circulating serelaxin. Serelaxin-mediated augmentation of bradykinin-evoked relaxation involved endothelium-derived hyperpolarization after 3 hours and prostacyclin-mediated relaxation after 24 hours. Furthermore, upregulation of inducible nitric oxide synthase, phosphorylation of protein kinase B at Ser473 and endothelial nitric oxide synthase at Ser1177 was observed at 24 hours after serelaxin injection. There were no effects of serelaxin on passive arterial wall stiffness.

Conclusion—Our data show that a bolus intravenous injection of serelaxin modulates endothelial vasodilator function 3 hours after administration, an effect that was sustained for 24 hours. The prolonged bradykinin-mediated vasorelaxation is principally mediated through prostacyclin. (*J Am Heart Assoc.* 2014;3:e000493 doi: 10.1161/JAHA.113.000493)

Key Words: endothelium • endothelium-derived hyperpolarization • nitric oxide • prostacyclin • serelaxin

The peptide hormone relaxin is a renal and systemic vasodilator, increases global arterial compliance, increases cardiac output, and mediates these parameters during pregnancy in conscious rats.^{1,2} These animal studies and later preclinical studies in compensated heart failure patients, which showed that relaxin decreases pulmonary capillary wedge pressure and systemic vascular resistance,^{3–5}

suggested that serelaxin treatment results in rapid vasorelaxation to improve the symptoms of heart failure. Indeed, a recent phase III clinical trial (RELAXin in Acute Heart Failure [RELAX-AHF]) demonstrated that a 48-hour infusion of serelaxin (the recombinant form of human relaxin-2) to patients admitted to hospital with acute heart failure resulted in significant beneficial clinical outcomes. Specifically, serelaxin improved dyspnoea relief, reduced in-hospital worsening of acute heart failure, and decreased cardiovascular and all-cause mortality at 180 days.⁶

Receptors for relaxin, RXFP1, are expressed in arteries and veins and localized to both vascular smooth muscle cells and the endothelium.^{7–9} Many studies have proposed an endothelium-dependent mechanism to explain the vasodilator actions of continuously infused relaxin on small renal arteries. This involves increased vascular activity of matrix metalloproteinases (MMP 2 and MMP 9), vascular endothelial growth factor, and placental growth factor, leading to activation of endothelin B receptors and nitric oxide (NO)-dependent vasodilation.^{10–13} Furthermore, a direct ex vivo vasodilator response to relaxin has been demonstrated in human small resistance

From the Departments of Zoology (C.H.L., M.J., L.J.P.) and Physiology (M.J.), The University of Melbourne, Parkville, VIC, Australia; Department of Physiology, Monash University, Clayton, VIC, Australia (H.C.P., M.T.).

Accompanying Figures S1 through S4 are available at <http://jaha.ahajournals.org/content/3/1/e000493/suppl/DC1>

Correspondence to: Laura J. Parry, PhD, Gate 12, Department of Zoology, The University of Melbourne, Parkville, VIC, 3010 Australia. E-mail: ljparry@unimelb.edu.au

Received December 16, 2013; accepted January 10, 2014.

© 2014 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley Blackwell. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

arteries.^{14,15} This is mediated by a rapid phosphatidylinositol-3-kinase (PI3K) stimulation of protein kinase B (Akt) and endothelial nitric oxide synthase (NOS) phosphorylation leading to increased endothelium-derived NO production.¹⁵ Relaxin also increases coronary flow in rat and guinea pig hearts via stimulation of NO production.¹⁶ The endothelium is an important regulator of vascular tone, which in small resistance arteries is influenced by endothelium-derived hyperpolarization (endothelium-derived hyperpolarizing factor [EDH])¹⁷ and the release of NO and prostacyclin (PGI₂).¹⁸ However, no studies to date have investigated if the actions of relaxin in the systemic vasculature involve a contribution of PGI₂ and EDH.

Time-course studies in preclinical models report that an intravenous (IV) relaxin injection into the femoral vein followed by long-term infusion increased glomerular filtration rate within 1 to 2 hours, which is indicative of rapid onset of renal vasodilation.¹⁹ The response to relaxin persisted in the kidneys for 12 hours, but not at 24 hours, after infusion of relaxin had ceased. However, the concentration of circulating relaxin in the plasma was not reported; thus, it is unclear if the sustained change in glomerular filtration rate in response to relaxin treatment occurred in the presence or absence of detectable circulating relaxin.¹⁹

There has been no preclinical assessment of the vascular effects of a single bolus injection of serelaxin and the persistence of a vasorelaxation response. Therefore, the aims of the present study were to investigate changes in vascular reactivity and passive mechanical wall properties of small mesenteric arteries following a bolus IV injection of serelaxin *in vivo* and to investigate whether these vascular effects are sustained in the absence of detectable circulating serelaxin. We also tested the hypothesis that in this small resistance artery, the contribution of EDH and PGI₂ to endothelium-dependent relaxation is enhanced by serelaxin treatment.

Methods

All procedures were approved by the Faculty of Science Animal Experimentation Ethics Committee (The University of Melbourne) and conformed to the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes.

Intravenous Injection of Serelaxin

Male Wistar rats (≈300 to 400 g; Animal Resource Centre) were randomly divided into 3 groups: (1) controls (n=47), (2) 3 hours (n=31) after serelaxin, and (3) 24 hours (n=26) after serelaxin treatment. These 2 time points were chosen based on pharmacokinetic modeling of serelaxin clearance *in vivo*, which showed that circulating serelaxin levels persist at 3 hours but not at 24 hours after serelaxin treatment (data not shown). For

drug administration, all animals were briefly anesthetized with 2% isoflurane and then maintained at 1% (Univentor 400; Agnethos) in oxygen mix via inhalation. Once sedated, the rats were placed on a heating pad (43°C), and a single bolus dose of serelaxin (13.3 μg/kg) was injected into the tail vein, which is equivalent to the standard infusion dosage of 4 μg/h.^{13,19} The control groups received an equivalent volume of the placebo (20 mmol/L sodium acetate). At either 3 or 24 hours post serelaxin injection, blood samples were obtained from the left ventricle via cardiac puncture under 2% isoflurane anesthesia. Plasma concentrations of serelaxin were measured using the Human Relaxin-2 Quantinine ELISA Kit (R&D Systems).

Isolation of Mesenteric Arteries

After blood collection, the animals were killed via cervical dislocation. The mesenteric arcade was isolated and immediately placed in ice-cold Krebs bicarbonate solution containing (in mmol/L) NaCl 120, KCl 5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, D-glucose 11.1, and CaCl₂ 2.5, bubbled with 95% O₂ and 5% CO₂. Small mesenteric arteries (third-order branch of the superior mesenteric artery, internal diameter ≈300 μm) were isolated, cleared of fat and loose connective tissue, cut into rings 2 mm in length, and mounted on a Mulvany-Halpern wire-myograph (model 610M; Danish Myo Technology). The remaining arteries were snap frozen in liquid nitrogen and stored at −80°C for further analysis. After the arteries were mounted on the myograph, the vessels were allowed to stabilize at zero tension for 15 minutes before normalization, as described previously.^{21,22} All experiments were performed at 37°C, and the baths were bubbled with 95% O₂ and 5% CO₂.

Assessment of Vascular Reactivity

Vascular reactivity was performed as previously described^{23,24} with the following modifications. Briefly, mesenteric arteries were maximally contracted with high K⁺ physiological saline solution (KPSS, 100 mmol/L), and the integrity of the endothelium was determined, as described previously.²⁵ To evaluate the vascular smooth muscle reactivity to vasoconstrictors, cumulative concentration-response curves to endothelin-1 (ET-1, 0.1 nmol/L to 0.1 μmol/L) and the thromboxane mimetic, U46619 (0.1 nmol/L to 1 μmol/L), were constructed. In addition, constrictor responses to ET-1 were examined after 20 minutes incubation with N ω -nitro-L-arginine methyl ester (L-NAME, 200 μmol/L), a NOS inhibitor and in endothelium-denuded vessels. Acute vasodilator actions of serelaxin were determined by using cumulative concentration-response curves to serelaxin (10 pmol/L to 0.3 μmol/L) in phenylephrine (0.1 to 3 μmol/L)-precontracted mesenteric arteries. Similarly, to assess endothelial and vascular smooth muscle function, mesenteric arteries were

precontracted to a similar level (70% to 80% of KPSS contraction) using phenylephrine (0.1 to 3 $\mu\text{mol/L}$), and cumulative concentration-response curves to the endothelium-dependent agonists acetylcholine (ACh; 0.1 nmol/L to 10 $\mu\text{mol/L}$) and bradykinin (BK; 0.1 nmol/L to 1 $\mu\text{mol/L}$), intermediate conductance calcium-activated potassium channel (IK_{Ca}), small conductance calcium-activated potassium channel (SK_{Ca}) opener NS309 (1 nmol/L to 10 $\mu\text{mol/L}$), endothelium-independent agonists sodium nitroprusside (SNP, 0.01 nmol/L to 10 $\mu\text{mol/L}$) and iloprost (1 pmol/L to 0.1 $\mu\text{mol/L}$) were determined. In addition, responses to BK were examined after 20 minutes of incubation with different combinations of pharmacological blockers, including L-NAME (200 $\mu\text{mol/L}$), the cyclooxygenase (COX) inhibitor indomethacin (Indo, 1 $\mu\text{mol/L}$), the IK_{Ca} inhibitor TRAM34 (1 $\mu\text{mol/L}$), the SK_{Ca} inhibitor apamin (1 $\mu\text{mol/L}$), and a PGI_2 synthase inhibitor U51605 (1 $\mu\text{mol/L}$). The residual relaxation after blockade of NOS and COX is attributed to EDH.²⁶ The relative contribution of NO, PGI_2 , and EDH to relaxation evoked by BK was determined by analyzing the area under the curve (AUC) of the BK-response curve.²⁷ In brief, the response attributable to the PGI_2 was calculated by subtracting AUC in the presence of Indo+L-NAME from that obtained in L-NAME alone. Similarly, the component of the response mediated by NO was determined by subtracting the AUC in L-NAME from the AUC obtained in the absence of inhibitors. The AUC that remained in the presence of Indo+L-NAME was attributed to EDH. Prostanoids have little or no contribution to ACh-induced relaxation in rat mesenteric artery.²⁸ Thus, for ACh-mediated relaxations, the component of the relaxation attributed to NO was determined by subtracting the AUC in the presence of Indo+L-NAME from the AUC obtained in the absence of inhibitors. Similarly, the component of relaxation mediated by EDH was the AUC that remained in the presence of Indo+L-NAME.

Assessment of Basal NOS Activity

In a separate set of experiments, the effect of serelaxin treatment on basal NO release was examined through the addition of L-NAME (200 $\mu\text{mol/L}$) in endothelium-intact rings submaximally contracted with phenylephrine (10 to 100 nmol/L) to $\approx 20\%$ of KPSS contraction. Under these conditions, a contractile response to L-NAME was considered to reflect the level of basal NOS activity.²⁹

Passive Mechanical Wall Properties

At 24 hours after serelaxin injection, mesenteric, femoral, and renal arteries were transferred to Ca^{2+} -free EGTA (2 mmol/L) containing Krebs-HEPES buffer (Ca^{2+} -free PSS), pH 7.4, until mounted on a pressure myograph (Living Systems Instrumentation). Leak-free segments (3 to 4 mm in length) of artery

were then mounted on a glass cannula (tip diameter $\approx 200 \mu\text{m}$) filled with Ca^{2+} -free PSS. The lumen was gently flushed to remove any remaining blood, and then the distal end was tied off to prevent flow. The arteries were bathed in Ca^{2+} -free PSS warmed to 37°C . After a 20-minute equilibration period, intraluminal pressure was increased from 0 to 190 mm Hg, in 10-mm Hg increments. The vessel length, outside diameter (OD), and wall thickness (WT) were measured at each pressure step. Inside diameter (ID) was calculated by subtracting the WT from the OD. Wall stress and strain were derived from the following calculations: wall stress (kPa)=(intraluminal pressure \times ID)/(2 \times WT); wall strain=(ID-ID extrapolated to 5 mm Hg pressure)/(ID extrapolated to 5 mm Hg pressure), as described previously.⁷ For normalization of ID and OD, values were expressed as: (value at current pressure–value at 5 mm Hg)/(value at 5 mm Hg). Volume compliance was calculated for each pressure increment using the following calculation: volume compliance=(Δ volume)/(Δ pressure), where Δ volume=(Δ cross-sectional area) \times (Δ length), where cross-sectional area=($\pi \times \text{ID}^2$)/4.

Western Blotting

Western blots were performed as described previously²³ with the following modifications. Frozen endothelium-intact mesenteric arteries were placed in a prechilled Wig-L-Bug capsule with a metal silver ball and pulverized in a Digital Wig-L-Bug amalgamator (Dentsply Ltd). The samples were dissolved in 300 μL of ice-cold lysis buffer (100 mmol/L NaCl, 10 mmol/L Tris, 2 mmol/L EDTA, 0.5% w/v sodium deoxycholate, 1% vol/vol Triton X-100, pH 7.4, protease, and phosphatase inhibitor cocktails (Roche)), and the total protein concentration of the samples was quantified using a BCA protein assay kit (ThermoScientific). Equal amounts of protein homogenate were subjected to SDS-PAGE and Western blot analysis with mouse/rabbit primary antibodies (all 1:1000, overnight, 4°C) against Akt, phospho-Ser473 Akt (all Cell Signalling) and inducible NOS (iNOS), endothelium NOS (eNOS), and phospho-Ser1177 eNOS (all BD Transduction Laboratories). To normalize for the amount of protein, membranes were reprobed with a loading control antibody (actin). All proteins were detected by using enhanced chemiluminescence (Amersham, GE Healthcare Life Sciences) after incubation with anti-mouse/rabbit secondary antibody (Millipore) for 1 hour at room temperature (1:2000). All protein bands were quantified by using densitometry (Biorad Chemidoc) and expressed as a ratio of the loading control.

RNA Extraction and Quantitative Polymerase Chain Reaction

Frozen blood vessels were pulverized as described here earlier. Pulverized tissues were resuspended in 1 mL

TriReagent (Ambion Inc), and total RNA was then extracted according to the manufacturer's instructions. RNA pellets were resuspended in 15 to 20 μ L RNA Secure (Ambion). Quality and quantity of RNA were analyzed using the NanoDrop ND1000 Spectrophotometer (Thermo Fisher Scientific Australia Pty Ltd) with $A_{260}:A_{280}$ ratios greater than 1.8, indicating sufficient quality for quantitative real-time polymerase chain reaction (qPCR) analysis. First-strand cDNA synthesis used 1 μ g of total RNA in a 20- μ L reaction containing random hexamers (50 ng/ μ L) and 200 units of Superscript III (Invitrogen). The comparative cycle threshold ($2^{-\Delta C_T}$) method of qPCR was used to analyze cyclooxygenase 1 (*Ptgs1*) and 2 (*Ptgs2*), PGI₂ synthase (*Ptgis*), and receptor (*Ptgir*), BK type 2 receptor (*Bdkrb2*, *Bdkrb2v1*), and eNOS (*Nos3*) gene expression in serelaxin and placebo-control rats. Rat-specific forward/reverse primers and 6-carboxyl fluorescein-labeled (FAM) Taqman probes were designed and purchased from Biosearch Technologies. Primers were designed to span intron/exon boundaries. qPCR was performed on the AB Applied Biosystems ViiA7 PCR machine (Life Technologies) using 96-well reaction plates with 10- μ L volume reactions in triplicate containing SensiMix (Bioline) and 10 μ mol/L primers and FAM-labeled probe. Ribosomal 18S (*Rn18s*) was the reference gene. 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. For each sample, the mean *Rn18s* 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 presented as a relative value (mean \pm SEM).

Reagents

All drugs were purchased from Sigma-Aldrich, except for ET-1 and BK (Auspep) and U46619 (Cayman Chemical). All drugs were dissolved in distilled water, with the exception of Indo, which was dissolved in a 0.1 mol/L sodium carbonate, and U46619, which was dissolved in 100% ethanol (final concentration <0.1% ethanol) as 1 mmol/L stock solution, and subsequent dilutions were in distilled water.

Statistical Analyses

All results are expressed as the mean \pm SEM, and n represents the number of animals per group or the number of assays when tissues from animals were pooled. Concentration-response curves for rat mesenteric arteries were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 5.0; GraphPad Software) to calculate the sensitivity of each agonist (pEC_{50}). Concentration-response curves were also analyzed with the use of repeated-measures 2-way

ANOVA (treatment versus concentration). Maximum relaxation (R_{max}) to vasodilators was measured as a percentage of precontraction to phenylephrine. Group pEC_{50} and R_{max} values were compared via 1-way ANOVA with post-hoc analysis using Dunnett's test or Student's unpaired t test as appropriate. Stress-strain curves were analyzed with the use of repeated-measures 2-way ANOVA (treatment versus strain). $P<0.05$ was considered statistically significant.

Results

Serelaxin Reduced ET1-Mediated Contraction 3 Hours After IV Injection in the Mesenteric Artery

Plasma serelaxin was detected 3 hours after serelaxin injection (range 1.23 to 4.17 ng/mL, mean 2.33 ng/mL) but was undetectable after 24 hours. Contraction evoked by KPSS (100 mmol/L) was not affected by serelaxin treatment at either time point (Figure S1). The direct vasorelaxant effects of serelaxin are shown in Figure S2. Responses to cumulative addition of serelaxin were not significantly different from the time control, indicating that acute serelaxin application did not produce a relaxation in the rat mesenteric artery. Maximum contraction, but not sensitivity to ET1, was significantly ($P<0.001$) reduced by ≈ 1.35 -fold 3 hours after serelaxin injection (Figure 1A). Suppression of the ET1 contraction was not sustained, and by 24 hours after serelaxin treatment, contraction amplitude was comparable to that of mesenteric arteries from placebo-treated rats. To investigate if NO released from the endothelium suppressed maximum contraction to ET1 3 hours after serelaxin injection, ET1 responses were evaluated in the presence of L-NAME and in endothelium-denuded mesenteric arteries. The maximum contraction to ET1 was not different between placebo and serelaxin-treated groups after blockade of NOS (Figure 1B) and endothelium removal (Figure 1C), confirming that endothelium-derived NO mediated the reduction in the maximum contraction to ET1 3 hours after serelaxin injection.

IV Serelaxin Injection Selectively Improved BK-Evoked Endothelium-Dependent Relaxation in the Mesenteric Artery

Endothelium-dependent relaxation is mediated by multiple factors, including NO,^{30,31} PGI₂,¹⁸ and EDH.¹⁷ To evaluate whether serelaxin enhances endothelial vasodilator function, vascular reactivity to 2 different endothelium-dependent agonists, ACh and BK, were determined. There was no significant effect of serelaxin on the sensitivity and maximum relaxation to ACh at either 3 or 24 hours after injection (Figure 2A). In contrast, the sensitivity, but not maximum relaxation, to BK was significantly increased by ≈ 4.5 -fold

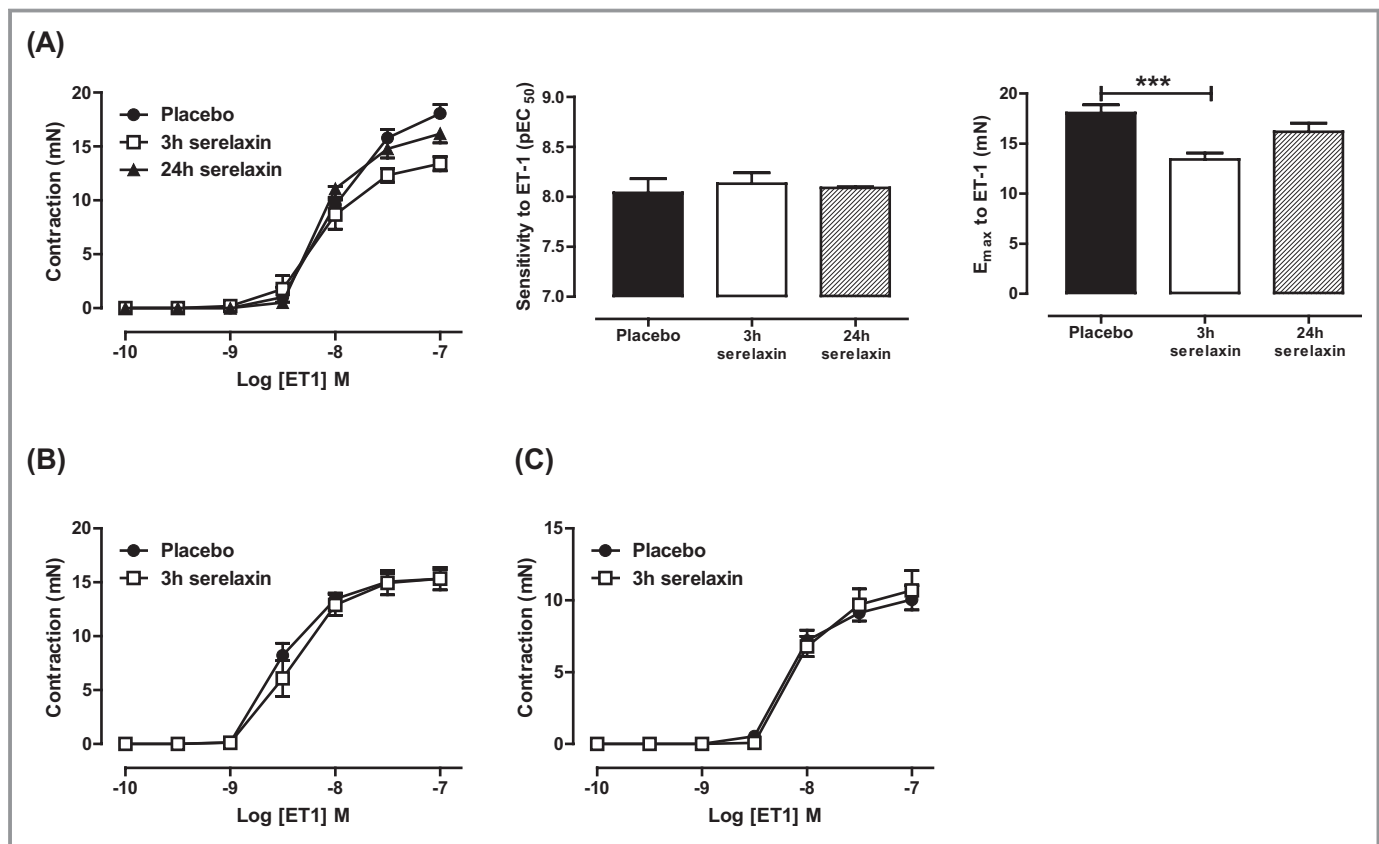


Figure 1. At 3 hours post serelaxin treatment, reduced ET1 induced contraction through endothelium-derived NO pathway. Concentration-response curves, sensitivity (pEC₅₀), and maximal responses to ET1. Responses in the (A) absence or (B) presence of L-NAME in endothelium-intact mesenteric and (C) endothelium-denuded arteries isolated from (●) placebo, (□) at 3 hours, and (▲) at 24 hours after a single bolus injection of serelaxin (n=4 to 8 per group). ***P<0.001 (1-way ANOVA, Dunnett's post-hoc test). ET1 indicates endothelin-1; L-NAME, N ω -nitro-L-arginine methyl ester; NO, nitric oxide; pEC₅₀, negative log of half-maximal effective concentration.

after 3 hours ($P<0.01$) and by ≈ 6.08 -fold at 24 hours ($P<0.001$) after serelaxin injection (Figure 2B).

To explore whether serelaxin injection alters the mechanism of ACh-induced relaxation, vascular reactivity to ACh was evaluated in the presence of the combination of COX and NOS inhibitors Indo+L-NAME. In all groups, the presence of these inhibitors significantly shifted the ACh-response curve to the right (Figure S3A) without affecting maximum relaxation to ACh. Because there is little or no contribution of PGI₂ to ACh-mediated endothelium-dependent relaxation in the rat mesenteric artery,²⁸ we only assessed the contribution of NO and EDH. In the presence of Indo+L-NAME, the sensitivity (Figure S3B) and maximum relaxation (Figure S3C) to ACh were similar between the groups of rats, indicating that serelaxin had no effect on the contribution of EDH to ACh-induced relaxation. Similarly, AUC analysis of ACh-response curves revealed that the relative contribution of NO and EDH in ACh-evoked relaxation was unaffected by serelaxin treatment (Figure S3D).

We then investigated endothelial factors associated with the enhanced BK-induced relaxation after serelaxin injection. Responses to BK were obtained in the presence of either

L-NAME alone or the combination of Indo+L-NAME. In all groups, including the placebo, the presence of L-NAME alone or the combination of Indo+L-NAME significantly shifted the BK-response curve to the right (Figure 3A). Thus, the sensitivity to BK was significantly reduced, but maximum relaxation was unaltered in mesenteric arteries. Blockade of COX activity had no effect on the BK-response curve in rats treated with placebo or 3 hours after serelaxin injection. However, at 24 hours after serelaxin, the addition of Indo to L-NAME caused a significant rightward shift of the BK-response curve compared with L-NAME alone (Figure 3A). This suggests a role of vasodilator prostanoids in mesenteric arteries 24 hours after serelaxin treatment.

In the presence of L-NAME, the sensitivity to BK was significantly ($P<0.05$) increased in arteries at 3 and 24 hours after serelaxin injection compared with those from placebo-treated rats. In the presence of Indo and L-NAME, the sensitivity to BK remained significantly ($P<0.05$) increased 3 hours after serelaxin (Figure 3B), indicating that serelaxin treatment improves BK-dependent EDH-type relaxation. Conversely, the sensitivity to BK after 24 hours serelaxin was

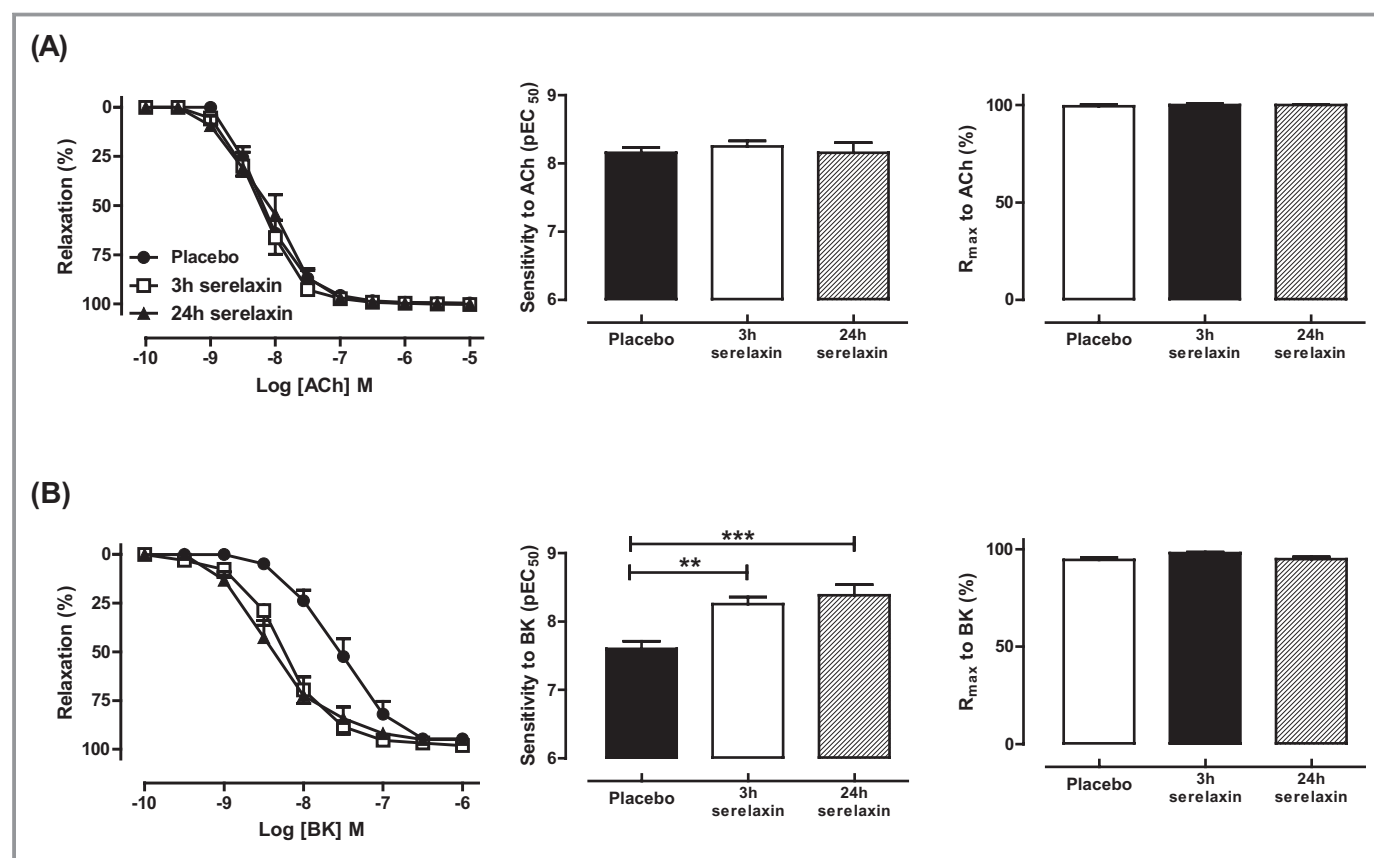


Figure 2. Serelaxin treatment selectivity enhances sensitivity to BK-induced relaxation. Concentration–response curves, sensitivity (pEC_{50}), and maximum relaxation (R_{max}) to (A) ACh and (B) BK in endothelium-intact mesenteric arteries isolated from (●) placebo, (□) at 3 hours, and (▲) at 24 hours after a single bolus injection of serelaxin ($n=8$ to 10 per group). $**P<0.01$, $***P<0.001$ (1-way ANOVA, Dunnett's post-hoc test). ACh indicates acetylcholine; BK, bradykinin; pEC_{50} , negative log of half-maximal effective concentration.

attenuated to a level that was comparable to that of placebo-treated rats (Figure 3B) with the blockade of NOS and COX, indicating no prolonged involvement of EDH. Analysis of the AUC of the BK-response curves to reveal the relative contribution of NO, EDH and COX also demonstrated that EDH-type and COX-mediated relaxation were augmented at 3 and 24 hours after serelaxin injection (Figure 3D).

In a separate series of experiments, the relative contribution of IK_{Ca} and SK_{Ca} channels underlying the augmented BK-evoked EDH-type relaxation was investigated 3 hours after serelaxin injection using TRAM34 and apamin, respectively. The sensitivity of BK was significantly ($P<0.05$) increased 3 hours after serelaxin injection in the absence of inhibitors (Figure 4A) and in the presence of Indo+L-NAME (Figure 4B). In contrast, the sensitivity and maximum relaxation to NS309 were not significantly altered 3 hours after serelaxin injection (Figure 4C). In the presence of Indo+L-NAME+apamin, the sensitivity (pEC_{50} 7.74 ± 0.24 versus 7.04 ± 0.14) to BK was significantly ($P<0.05$) increased 3 hours after serelaxin injection (Figure 4D). Conversely, in the presence of Indo+L-NAME+TRAM34, maximum relaxation (R_{max} $26\pm17\%$ versus $73\pm11\%$) to BK was significantly ($P<0.05$) reduced 3 hours

after serelaxin injection (Figure 4E). The relaxation response evoked by BK was abolished in both placebo- and serelaxin-treated mesenteric arteries in the combined presence of Indo+L-NAME+apamin+TRAM34 (Figure 4F).

The involvement of PGI_2 as the vasodilator prostanoid factor responsible for the improvement of BK-mediated relaxation at 24 hours after serelaxin injection was elucidated using a selective PGI_2 synthase inhibitor, U51605. In Figure 5A, sensitivity to BK was significantly ($P<0.05$) increased 24 hours after serelaxin injection, which was abolished by the presence of Indo (Figure 5B) and U51605 (Figure 5C). Similarly, in the presence of L-NAME, the sensitivity to BK remained significantly ($P<0.05$) increased 24 hours after serelaxin injection (Figure 5D) and was abolished in the presence of L-NAME+U51605 (Figure 5E). We further explored the mechanisms underlying the improvement of BK-dependent PGI_2 -mediated relaxation 24 hours after serelaxin injection by analyzing the expression of genes associated with PGI_2 synthesis pathways. There was no effect of serelaxin on COX2 (*Ptgs2*) or PGI_2 synthase (*Ptgis*, Figure 6A and 6B) expression in mesenteric arteries at either time points. COX1 (*Ptgs1*) mRNA expression was not

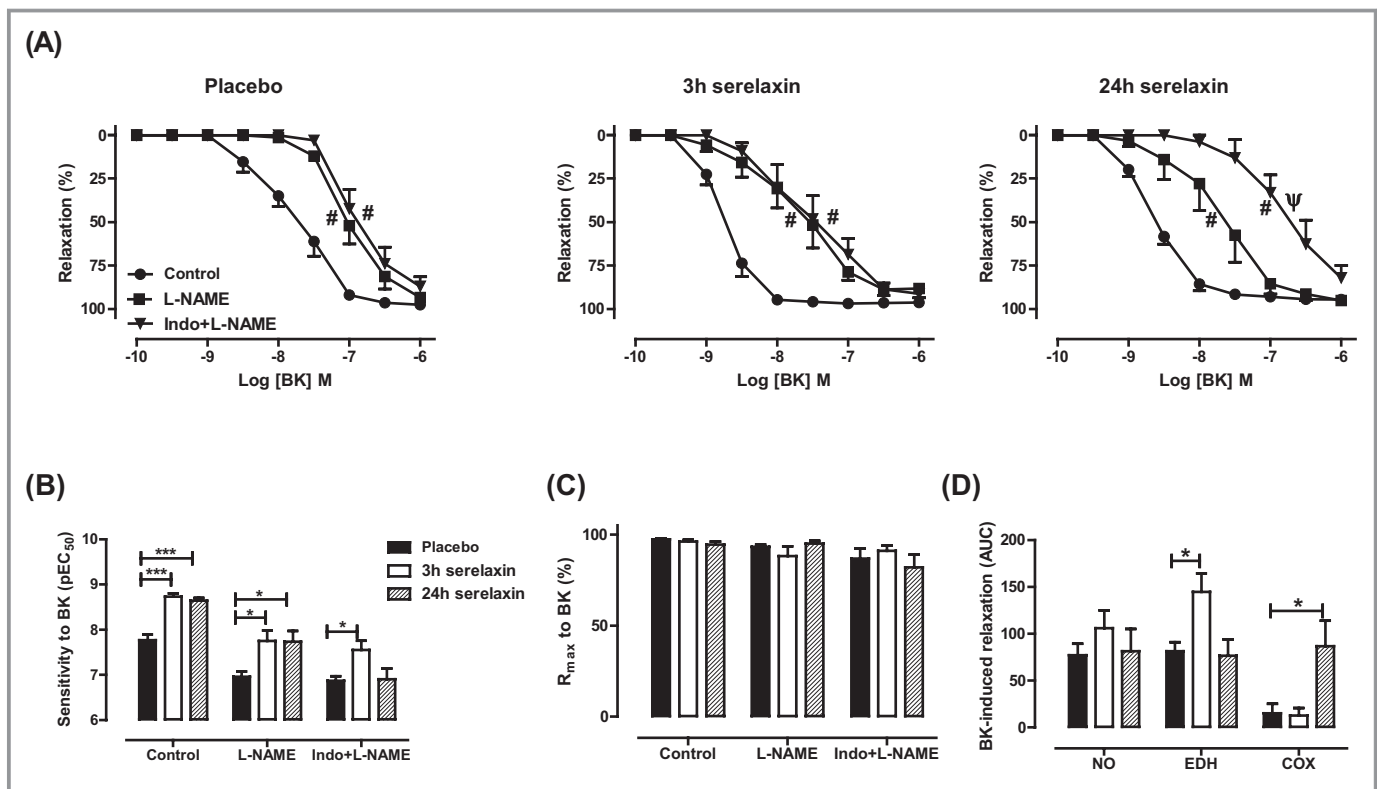


Figure 3. Serelaxin treatment causes time-dependent augmentation of the contributions of EDH and PGI₂ to BK-induced relaxation. Concentration–response curves to BK in the absence (control, ●) or presence of L-NAME (■, NOS inhibitor), Indo+L-NAME (▲, inhibitors of COX and NOS) in endothelium-intact mesenteric arteries isolated from (A) placebo, at 3 hours, and at 24 hours after a single bolus injection of serelaxin-treated rats. #*P*<0.05 vs control, Ψ*P*<0.05 vs L-NAME (2-way repeated-measures ANOVA). Mesenteric (B) sensitivity (pEC₅₀) and (C) maximum relaxation (R_{max}) to BK in (filled bars) placebo, (open bars) at 3 hours, and (striped bars) at 24 hours after a single bolus injection of serelaxin. Area under curve (D) of BK-response curve reveals the relative contribution of NO, EDH, and COX (n=6 to 7 per group). **P*<0.05, ****P*<0.001 (1-way ANOVA, Dunnett's post-hoc test). BK indicates bradykinin; COX, cyclooxygenase; EDH, endothelium-derived hyperpolarization; Indo, indomethacin; L-NAME, *N*-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; pEC₅₀, negative log of half-maximal effective concentration; PGI₂, prostacyclin.

determined ($C_T > 40$ cycles) in mesenteric arteries (data not shown). The enhancement of PGI₂-mediated response by serelaxin treatment could be due to an increase in the sensitivity to PGI₂ receptors and/or upregulation of their expression. As described earlier, smooth muscle sensitivity and maximum response to the PGI₂ analog iloprost were unaltered (Figure 8C). Similarly, serelaxin had no effect on *Ptgir* gene expression at both time points (Figure 6C). There was also no effect in BK type 2 receptors (*Bdkrb2v1* and *Bdkrb2*) after serelaxin treatment at either time point (Figure 6E and 6F).

IV Serelaxin Injection Increased Basal NOS Activity After 3 Hours but Produces Sustained Akt Phosphorylation After 24 Hours in the Mesenteric Artery

Basal NOS activity was assessed by measuring the contraction induced by L-NAME in arteries with a low

level of phenylephrine-induced tone.^{23,29} The L-NAME-induced contraction was significantly (*P*<0.05) greater in arteries 3 hours after serelaxin injection compared with those of placebo-treated rats (Figure 7A), indicating that basal NOS activity was increased. This effect was not observed in arteries 24 hours after serelaxin treatment. At 3 hours after serelaxin treatment, there was a significant (*P*<0.05) increase in phosphorylation of Akt^{ser473} in the mesenteric arteries that was sustained for 24 hours (*P*<0.001) (Figure 7B). Total Akt protein expression was not affected by serelaxin treatment at either time point (Figure 7C). Interestingly, phosphorylation of eNOS^{ser1177} (Figure 7D) and upregulation of iNOS protein (Figure 7F) in the mesenteric arteries was only significantly increased at 24 hours, but not at 3 hours, after serelaxin injection. Total eNOS gene (Figure 6D) and protein (Figure 7E) expression was not affected by serelaxin treatment at any time point.

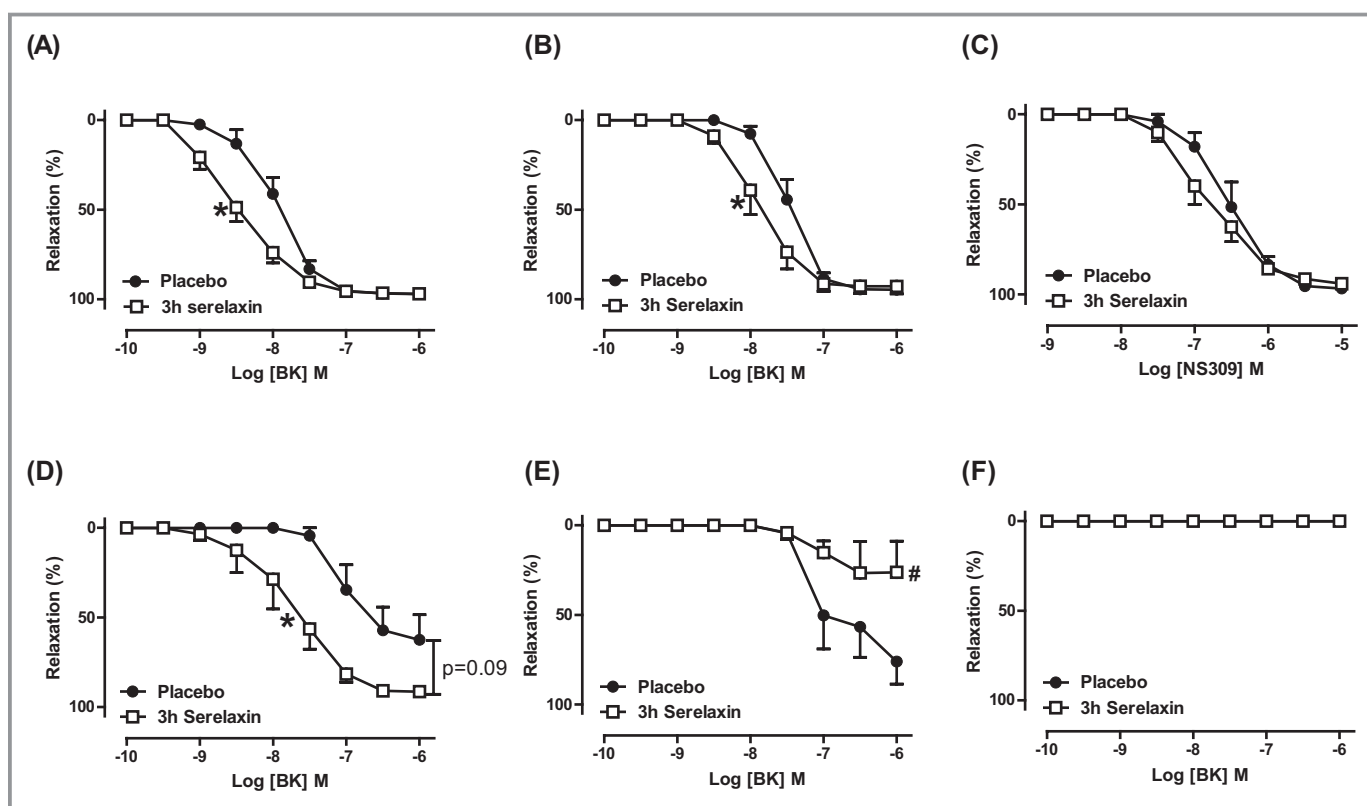


Figure 4. Involvement of IK_{Ca} channel in BK-induced EDH-type relaxation 3 hours after serelaxin injection. Concentration–response curves to BK in the (A) absence or (B) presence of Indo+L-NAME, (D) Indo+L-NAME+apamin, (E) Indo+L-NAME+TRAM34, and (F) Indo+L-NAME+TRAM34+apamin in endothelium-intact mesenteric arteries isolated from (●) placebo and (□) at 3 hours after a single bolus injection of serelaxin. Concentration–response curves to (C) NS309 in the endothelium-intact mesenteric arteries isolated from (●) placebo and (□) at 3 hours after a single bolus injection of serelaxin-treated rats ($n=6$ to 10 per group). * pEC_{50} significantly different from placebo, $P<0.05$ #maximum relaxation significantly different from placebo ($P<0.05$) (1-way ANOVA, Dunnett's post-hoc test). BK indicates bradykinin; EDH, endothelium-derived hyperpolarization; IK_{Ca} , intermediate conductance calcium-activated potassium channel; Indo, indomethacin; L-NAME, *N*-nitro-L-arginine methyl ester; TRAM34, 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole) pEC_{50} , negative log of half-maximal effective concentration.

IV Serelaxin Injection Had No Effect on Endothelium-Independent Vasoconstrictor and Vasodilator Function

Injection with serelaxin had no effect on the sensitivity or maximal response to the vasoconstrictor thromboxane mimetic U46619 (Figure 8A) at 3 hours, nor on the actions of the endothelium-independent vasodilators SNP (Figure 8B) at either time point, or by iloprost (Figure 8C) at 24 hours after a bolus IV injection.

IV Serelaxin Injection Had No Effect of Passive Mechanical Wall Properties of the Mesenteric, Saphenous, and Renal Arteries

Chronic infusion of serelaxin has been demonstrated to regulate vascular wall remodeling and passive mechanical wall properties.^{9,32} We sought to investigate whether this effect could be observed with a bolus injection of serelaxin. In

mesenteric arteries, serelaxin treatment had no significant effect on passive wall stiffness (Figure 9A) or volume compliance (Figure 9B) across the pressurization range compared with placebo-treated rats 24 hours after injection. Furthermore, there was no significant differences in OD (Figure 9C) or normalized OD, ID, and WT (Figure 9D). The effects of serelaxin on the passive stiffness of arteries from other vascular beds were also examined. As with the mesenteric arteries, the stress-strain relationships for saphenous and renal arteries from placebo- and serelaxin-treated rats were comparable (Figure S4), indicating there was no effect of serelaxin on passive wall stiffness.

Discussion

This study demonstrated that a single IV injection of serelaxin modulated endothelial vasodilator function 3 hours after administration. We suggest that serelaxin activated RXFP1 localized to endothelial cells and caused an increase in

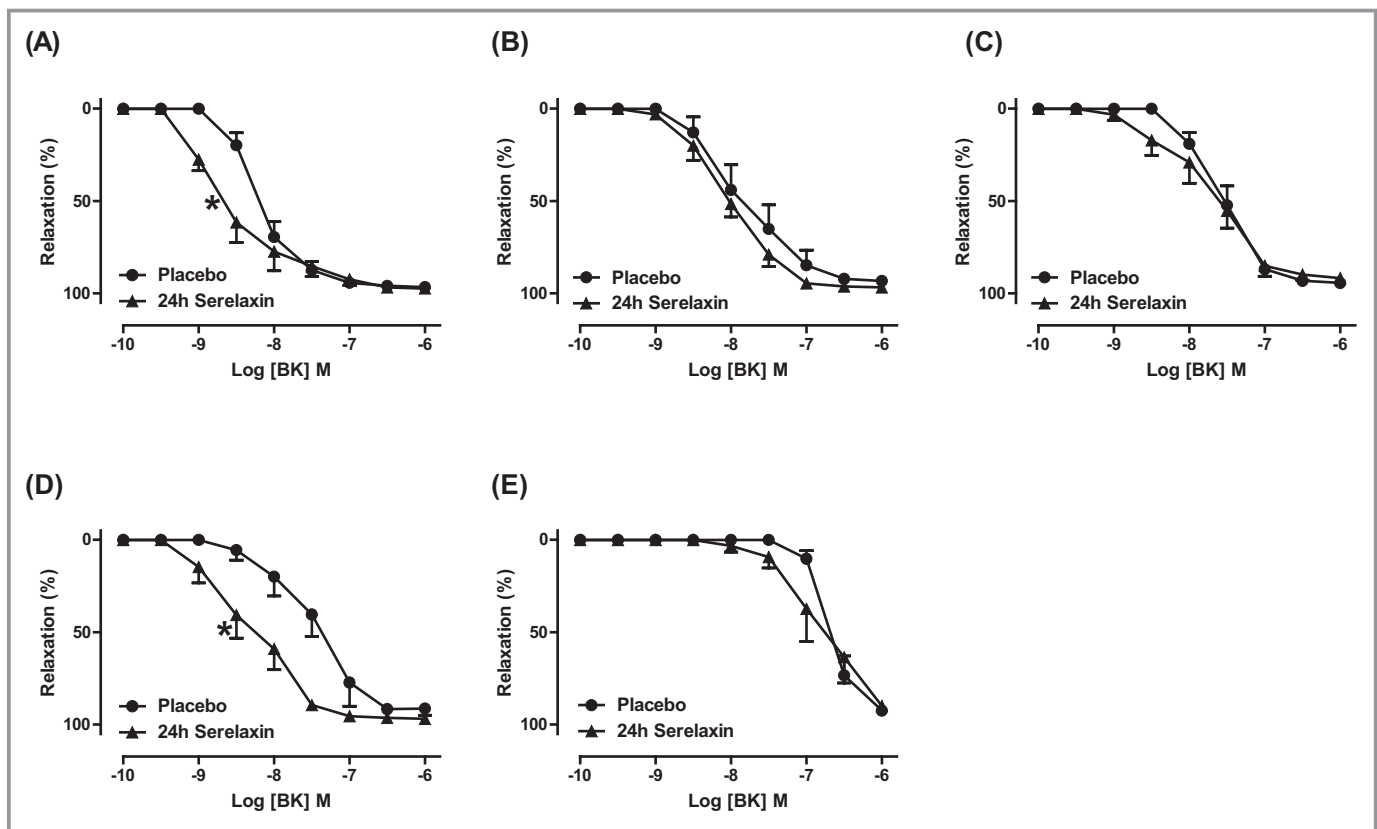


Figure 5. Involvement of PGI_2 in BK-induced relaxation 24 hours after serelaxin injection. Concentration–response curves to BK in the (A) absence or presence of (B) Indo, (C) U51605, (D) L-NAME, and (E) U51605+L-NAME in endothelium-intact mesenteric arteries isolated from (●) placebo and (▲) at 24 hours after a single bolus injection of serelaxin ($n=6$ to 7 per group). * pEC_{50} significantly different from placebo, $P<0.05$ (1-way ANOVA, Dunnett's post-hoc test). BK indicates bradykinin; Indo, indomethacin; L-NAME, *N* ω -nitro-L-arginine methyl ester; pEC_{50} , negative log of half-maximal effective concentration; PGI_2 , prostacyclin.

Akt^{Ser473} phosphorylation and basal NOS activity. Serelaxin also selectively enhanced BK-mediated endothelium-dependent relaxation in rat mesenteric arteries. Although circulating levels of serelaxin were not detected 24 hours after bolus injection, the effect on BK-mediated relaxation was sustained. Our findings indicate that the vasorelaxant effects of BK, which are potentiated by serelaxin, are mediated by different endothelium-derived relaxing factors over time. Specifically, serelaxin-mediated augmentation of BK-evoked relaxation involves IK_{Ca} -dependent EDH-type relaxation at 3 hours and PGI_2 -mediated relaxation at 24 hours. Furthermore, Akt remains phosphorylated at Ser473, with increased eNOS phosphorylation at Ser1177 and an upregulation in iNOS expression 24 hours after IV serelaxin injection.

Previous animal studies have shown a rapid vasodilator response to relaxin in resistance arteries,¹⁵ but it remains unclear how long these effects are sustained, especially to a bolus IV injection. Acute injection followed by long-term IV infusion of relaxin increased glomerular filtration rate, which was sustained for 12 hours, but not 24 hours, after treatment in rats.¹⁹ It is important to note that in that study, serum

levels of relaxin between 1 and 6 ng/mL produced physiological changes in the kidney, suggesting that our reported concentration of relaxin (2.33 ng/mL) was sufficient to produce a vasorelaxation. However, a direct effect of relaxin on the renal artery vascular function after a bolus relaxin injection was not explored. In the present study, we demonstrated that a single IV injection of serelaxin selectively improved endothelial function in the presence of circulating serelaxin (after 3 hours), as observed by an augmentation of endothelium-dependent relaxation, without affecting endothelium-independent relaxation. Furthermore, enhanced endothelial function was agonist specific, as serelaxin treatment had no effect on ACh-evoked responses but improved BK-mediated responses. In rat aortic rings treated in vitro with tumor necrosis factor- α , incubation with relaxin improved ACh-mediated relaxation.³³ This suggests that relaxin may have differential effects in conduit (aorta) versus resistance arteries (mesenteric artery) or that its actions are more pervasive under disease conditions. Although RXFP1 are expressed in the endothelial and vascular smooth muscle cells,^{7,9} the vascular actions of serelaxin after a single IV

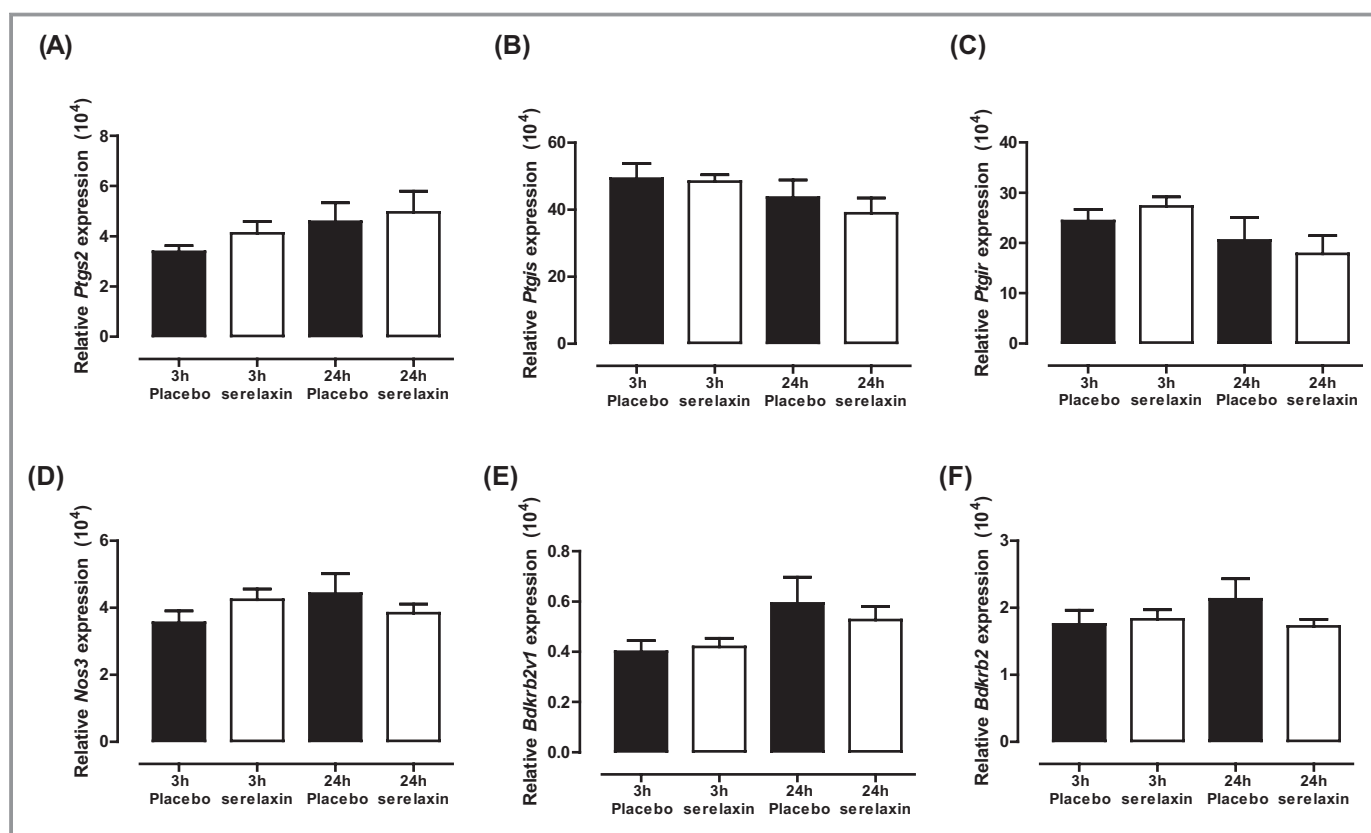


Figure 6. Serelaxin treatment had no effect on gene expression. Quantitative analysis of (A) *Ptgs2*, (B) *Ptgis*, (C) *Ptgir*, (D) *NOS3* (E) *Bdkrb2v1*, and (F) *Bdkrb2* expression in mesenteric arteries from rats at 3 and 24 hours after a single injection of (filled bars) placebo and (filled bars) serelaxin (n=7 to 8 per group). Values are $2^{-\Delta Ct} \pm \text{SEM}$. NOS indicates nitric oxide synthase.

injection appear mainly to be limited to the endothelium as serelaxin treatment had no effect on endothelium-independent relaxation (SNP and iloprost) and contraction (U46619).

The rapid vascular actions of relaxin are consistent with studies that have demonstrated that relaxin acts on RXFP1 on endothelial cells⁹ and causes endothelium-dependent relaxation of human and rodent renal arteries.^{14,15} However, it is important to note that the direct effect of relaxin is artery specific as serelaxin did not produce vasorelaxation in rat mesenteric arteries when applied cumulatively ex vivo in the organ bath. One proposed endothelium-dependent mechanism for the vasodilator action of relaxin involves activation of ET_B receptors and an increase in NO production in resistance arteries.³⁴ Our functional data also indicate that, 3 hours after serelaxin injection, there is a reduction in the maximum contraction to ET1. Consistent with earlier observations,³⁴ this effect was abolished by the NOS inhibitor L-NAME and endothelium removal, indicating that endothelium-derived NO was involved in the suppression of maximum contraction to ET1. In our study, we also demonstrated that at 3 hours after a single IV injection of serelaxin, there was an increase in basal NOS activity. This suggests that serelaxin may alter the activity of eNOS to increase production of NO. Activation of

eNOS is regulated by several protein kinases,³⁵ including Akt.³⁶ The level of phosphorylation of Akt on Ser473 was increased after 3 hours of serelaxin injection, without affecting total expression of Akt, eNOS, or, interestingly, eNOS^{ser1177}. Similarly, studies in cultured endothelial cells also demonstrated that serelaxin activates PI3K, leading to the phosphorylation of Akt^{ser473} and eNOS^{ser1177} and resulting in the generation of NO. Furthermore, 48 hours of relaxin treatment of organ-cultured rat aortic rings ex vivo augmented eNOS activity but not expression via enhanced phosphorylation at Ser1177 and Ser633 and dephosphorylation at Thr495.³³ Collectively, these observations indicate that 3 hours after a single injection of serelaxin, there were significantly increased levels of Akt phosphorylation, actions that would cause eNOS activation, probably through other eNOS phosphorylation sites (independent of eNOS^{ser1177}), and increase NO synthesis.³⁶

Despite the many reports showing that relaxin has sustained effects on the vascular system, the vasodilator effects of relaxin in vivo are always studied in the continuous presence of circulating relaxin.^{9,19,37} There is a paucity of information regarding sustained vascular function in the absence of circulating levels of relaxin. Thus, in the present

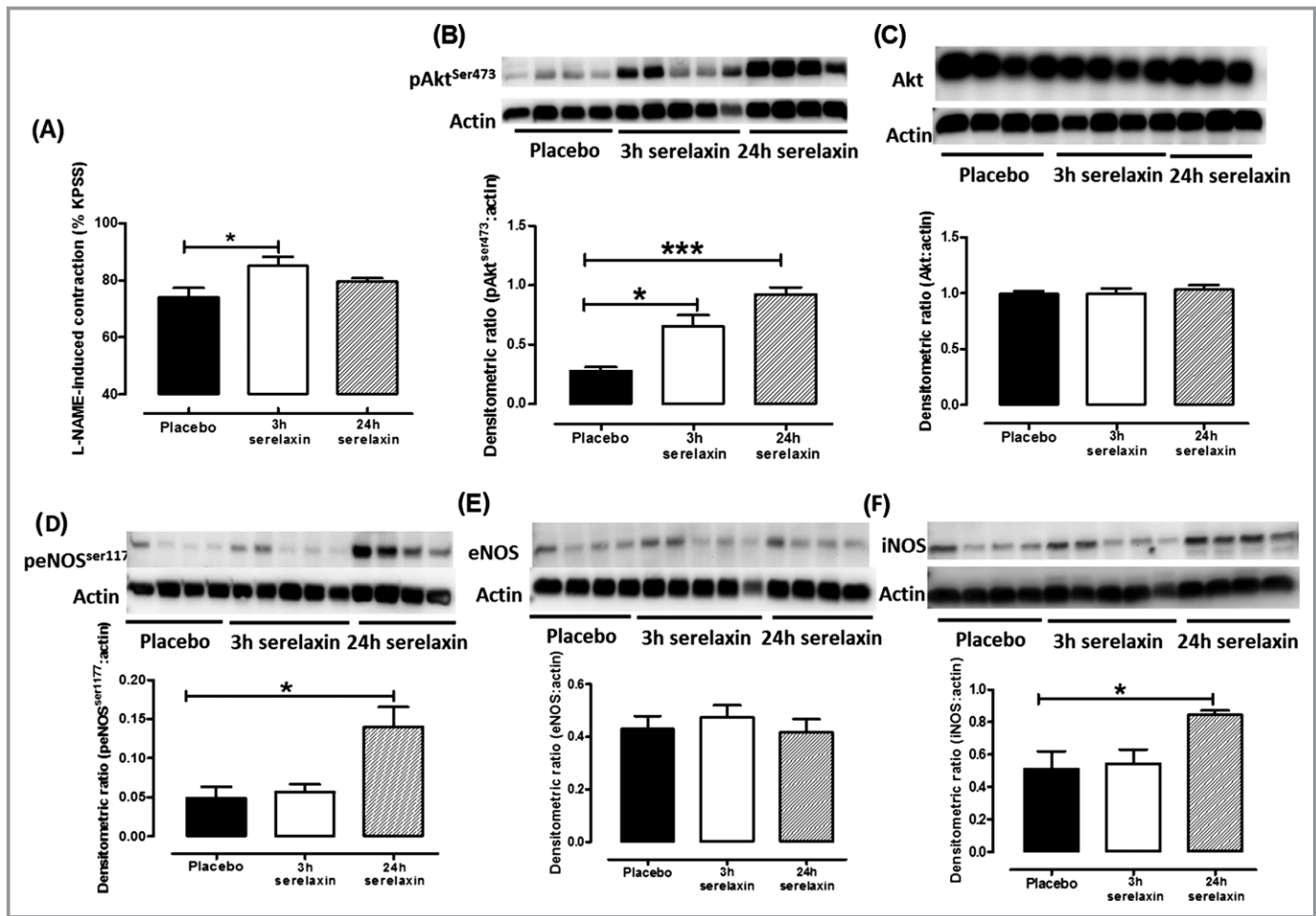


Figure 7. Serelaxin treatment increased basal NOS activity after 3 hours but produces sustained Akt phosphorylation from 3 to 24 hours in the mesenteric artery. (A) Basal NOS activity in endothelium-intact mesenteric arteries isolated from (filled bars) placebo, (open bars) at 3 hours, and (striped bars) at 24 hours after a single bolus injection of serelaxin. In each group of experiments, mesenteric arteries were precontracted with phenylephrine to similar levels (placebo $21 \pm 2\%$, 3 hours serelaxin $20 \pm 1\%$, and 24 hours serelaxin $22 \pm 1\%$ of KPSS) before the addition of L-NAME (NOS inhibitor) ($n=6$ to 8 per group). Western blot analysis of pAkt^{Ser473} (B, 60 kDa), total Akt (C, 60 kDa), peNOS^{Ser1177} (D, 140 kDa), total eNOS (E, 140 kDa), and iNOS (F, 140 kDa) protein expression in mesenteric arteries from (filled bars) placebo, (open bars) at 3 hours, and (striped bars) at 24 hours after a single bolus injection of serelaxin. Representative blots are shown in the figure ($n=4$ to 6 per group). * $P<0.05$, *** $P<0.001$ (1-way ANOVA, Dunnett's post-hoc test). Akt indicates protein kinase B; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; KPSS, K^+ physiological saline solution; L-NAME, *N*- ω -nitro-L-arginine methyl ester; NOS, nitric oxide synthase peNOS, phosphorylated NOS.

study, we evaluated vascular function of mesenteric arteries 24 hours after a single IV injection of serelaxin, where there is no detectable circulating level of serelaxin. Consistent with our earlier observation at the 3-hour time point, we found that, in the absence of serelaxin, there is augmentation of endothelium-dependent relaxation without affecting endothelium-independent relaxation. At the 24-hour time point, however, we do not observe any changes in ET1 responses or basal NOS activity, indicating that these effects may occur only in the presence of circulating serelaxin. Interestingly, the level of phosphorylation of Akt on Ser473 remains increased 24 hours after serelaxin injection, without affecting total Akt expression in the mesenteric artery. In addition, at this time point, there is an increase in phosphorylation of eNOS on

Ser1177 and upregulation of iNOS expression. Taken together, our data show that the direct and rapid effect of serelaxin on the endothelium requires circulating levels of serelaxin. However, serelaxin improves BK-mediated endothelium-dependent relaxation, an effect that is sustained in the absence of a circulating level of serelaxin.

Endothelium-dependent relaxation is mediated by multiple factors, including NO,^{30,31} PGI₂,¹⁸ and EDH.¹⁷ We further investigated the mechanisms underlying the augmentation of BK-mediated endothelium-dependent relaxation at 3 and 24 hours after a single bolus of serelaxin. Three hours after serelaxin treatment, when serelaxin is still present in the circulation, EDH-type relaxation is upregulated. However, at the 24-hour time point, when circulating levels of relaxin have

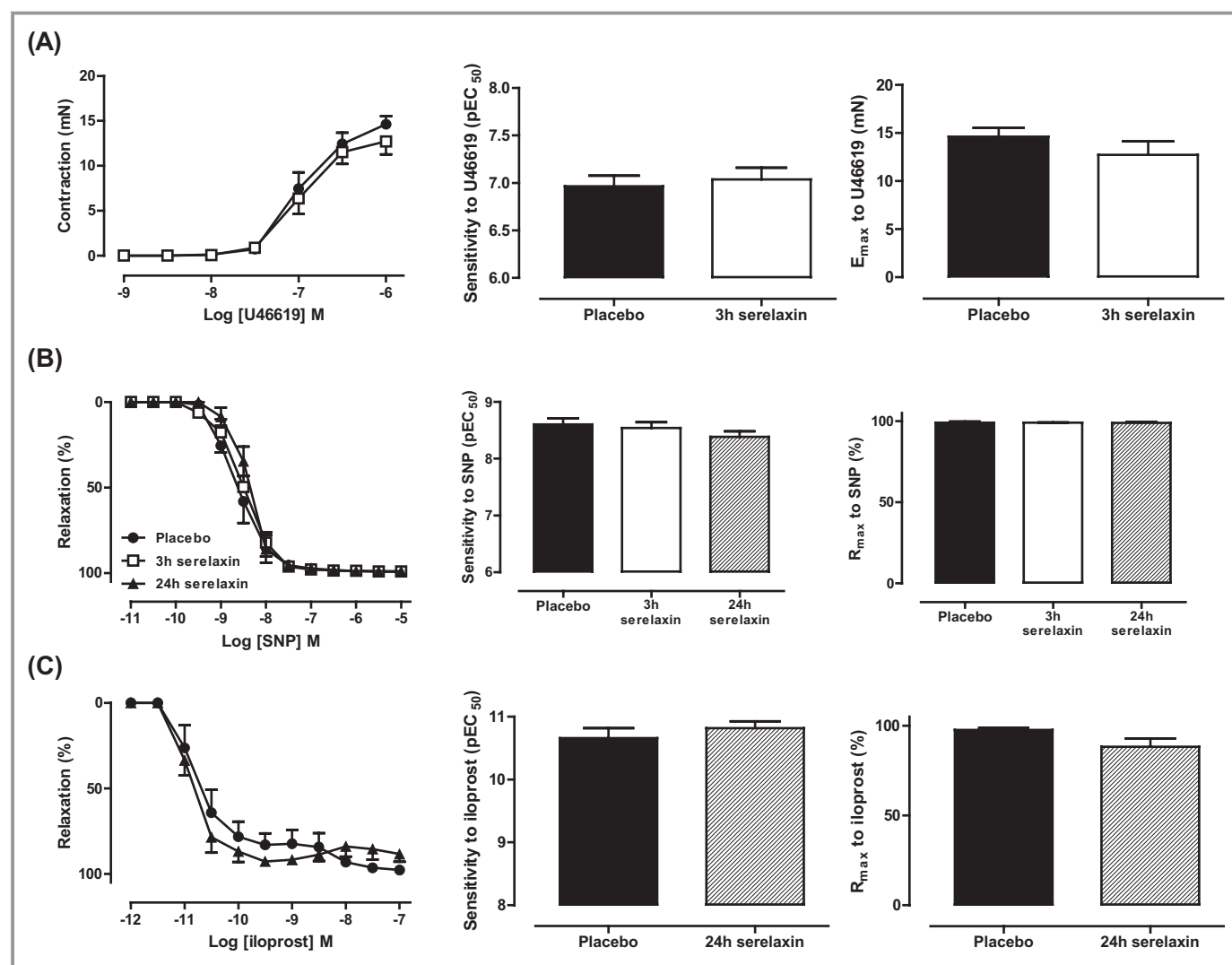


Figure 8. Serelaxin had no effect on endothelium-independent vasoconstrictor and vasodilator function. Concentration-response curves, sensitivity (pEC₅₀) and maximal responses to (A) U46619, (B) SNP, and (C) iloprost in endothelium-intact mesenteric arteries isolated from (●) placebo, (□) at 3 hours, and (▲) at 24 hours after a single bolus injection of serelaxin (n=5 to 8 per group). pEC₅₀ indicates negative log of half-maximal effective concentration; SNP, sodium nitroprusside.

declined below detection, the response attributed to a vasodilator prostanoid, presumably PGI₂ was augmented. In the rat mesenteric artery, EDH responses are generated by contact mediated (myoendothelial gap junctions) and non-contact-mediated mechanisms.^{26,38} Generation of EDH involves the opening of endothelial IK_{Ca} and SK_{Ca} channels and subsequent hyperpolarization and relaxation of smooth muscle cells.^{39–42} The relative contribution of IK_{Ca} and SK_{Ca} channels in responses to serelaxin treatment was elucidated using pharmacological inhibition. In the presence of apamin (Indo+L-NAME), the residual BK-induced relaxation is attributed to IK_{Ca} channels, and we demonstrated that BK response was significantly increased 3 hours after serelaxin injection. Similarly, in the presence of TRAM34 (Indo+L-NAME), the maximum relaxation to BK was reduced in mesenteric arteries

of serelaxin-treated rats, indicating that the upregulation of BK-evoked EDH-type relaxation involves IK_{Ca}, but not SK_{Ca}, channels. The combination of apamin and TRAM34 abolished BK-mediated relaxation, suggesting that BK-mediated EDH-type relaxation is entirely dependent on IK_{Ca} and SK_{Ca} channels. To date, there is limited evidence of the effects of relaxin on EDH-type responses. A recent study in rats⁴³ demonstrated that long-term treatment (10 days) with relaxin (circulating levels 58±8 ng/mL) suppressed myogenic tone in pressurized parenchymal arterioles via a mechanism involving IK_{Ca} channels. This suggests that long-term exposure to relaxin might be upregulating basal EDH responses. Although we examined EDH-mediated relaxation evoked by BK rather than the contribution of EDH to basal tone, both studies provide evidence that in the presence of circulating

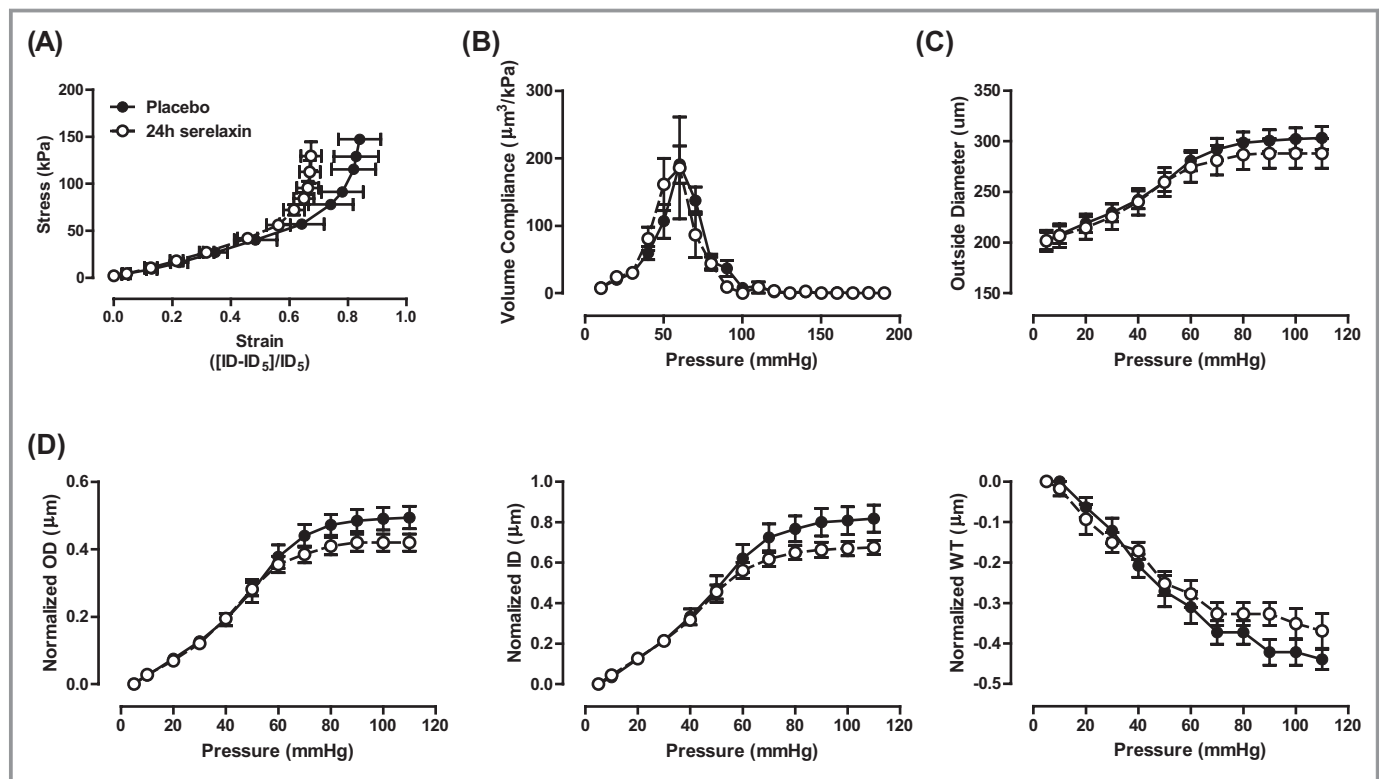


Figure 9. Serelaxin treatment had no effect on passive mechanical wall properties in the mesenteric artery. Stress-strain relationships (A) and relationships for volume compliance (B), outer diameter (C), and normalized inside (ID) and outside diameters (OD) and wall thickness (WT; D) against intraluminal pressures in mesenteric arteries isolated from (●) placebo and (○) at 24 hours after serelaxin. Values are mean±SEM (n=5 to 8 per group).

relaxin, the responses attributed to EDH are upregulated. Interestingly, responses to the opener of IK_{Ca} and SK_{Ca} channels, NS309, was not affected 3 hours after serelaxin injection, suggesting that the augmentation of EDH-type relaxation involved a signaling mechanism through the BK receptor, rather than activity of the IK_{Ca} and SK_{Ca} channels. This phenomenon is also reported in a recent study of SHR rats,⁴⁴ where long-term relaxin treatment had minimal effect in improving NS309 responses in hypertensive parenchymal arterioles. EDH is an important vasodilator mechanism in small arteries and arterioles and may be an additional mechanism by which relaxin reduces peripheral vascular resistance.

To determine the mechanism underpinning the prolonged action of serelaxin, vascular function was analyzed from rats at 24 hours post serelaxin treatment, where there was no detectable circulating level of serelaxin. Our data indicate upregulation of PGI_2 24 hours after serelaxin treatment, as this response was abolished by blockade of COX and PGI_2 S. Synthesis of PGI_2 involves a cascade of enzymes such as COX1, COX2, and PGI_2 synthase, which stimulates smooth muscle PGI_2 receptors, leading to accumulation of cAMP and vasodilation.^{18,45} Analysis of mRNA expression revealed that serelaxin treatment had no effect on *Ptgs2*, *Ptgs1*, or *Ptgir*.

Furthermore, serelaxin treatment did not enhance the sensitivity or maximum response to iloprost. Taken together, our findings suggest that the sustained vasorelaxant effect of serelaxin is likely to be mediated by endothelium-dependent mechanisms, possibly through increasing the activities of enzymes rather than expression. Because the mRNA expression of COX1 (*Ptgs1*) was not determined (expression too low) in the mesenteric arteries, it is likely that the augmentation of BK-induced PGI_2 -mediated relaxation is dependent on COX-2 expression/activity. Indeed, relaxin treatment for 24 hours has been shown to increase expression of COX2 in normal human endometrial stromal cells.⁴⁶ In our study, we demonstrated that Akt and eNOS were phosphorylated and that iNOS expression was upregulated in mesenteric arteries 24 hours after IV serelaxin injection, which could be due to Akt activation. This finding is also reported in a recent study,⁴⁷ which demonstrated that relaxin (5 μg/kg, IV) infusion protects against ischemia-reperfusion injury through the activation of Akt and upregulation of iNOS in the rat kidney.

It is well known that long-term subcutaneous relaxin infusion promotes compositional and geometric remodeling of blood vessels, resulting in increased vascular compliance in small renal, uterine, and mesenteric arteries.^{9,32,48,49}

Specifically, in mice treated with 1 µg/h relaxin for 5 days, there was an increase in unpressurized wall area and WT-to-lumen area ratio and smooth muscle cell density and a decrease in collagen-to-total protein in small renal artery.²⁰ In the present study, our data showed that 24 hours after a single injection of serelaxin, there was no effect on vessel dimensions and stress-strain relationships in mesenteric arteries, indicating that serelaxin had no short-term effect on passive wall stiffness. Similar observations were made in saphenous and renal arteries. Hence, we hypothesize that 24 hours is an insufficient time frame to cause sufficient vascular wall remodeling that would translate into changes in passive wall stiffness and compliance. Another possibility is that the duration of time and concentration of circulating serelaxin that is present after a single bolus dose of serelaxin may not be sufficient to stimulate vascular wall remodeling.

The present study has important clinical implications in that a single IV injection of serelaxin can elicit rapid effects on the endothelium and augment vasorelaxation actions of BK that are prolonged for at least 24 hours, even after circulating levels of relaxin have diminished. This initially involves EDH and NO, but the prolonged 24-hour response is attributed to PGI₂.

Acknowledgments

The authors thank Dr Dennis Stewart, the coordinator of the research partnership (Novartis Pharma AG, Basel, Switzerland), for helpful advice with the experimental design and critical analysis of the data. We also thank Dr Elaine Unemori for helpful suggestions and manuscript review.

Source of Funding

The research was funded by the Australian Research Council Linkage Grant (L.J. Parry, M. Tare, and H.C. Parkington) and an Australian and New Zealand Medical Research and Technology in Victoria Grant (L.J. Parry). Part of this research was also funded by Kaye Merlin Brutton Bequest (C.H. Leo). M. Jelinic received an Australian Postgraduate Award.

Disclosures

The authors disclose that this project was partially funded by Novartis Pharma AG, who also provided the serelaxin as a condition of an Australian Research Council Linkage Grant. Parry was also a paid consultant for Novartis Pharma AG.

References

- McGuane JT, Debrah JE, Debrah DO, Rubin JP, Segal M, Shroff SG, Conrad KP. Role of relaxin in maternal systemic and renal vascular adaptations during gestation. *Ann N Y Acad Sci*. 2009;1160:304–312.
- Conrad KP. Maternal vasodilation during pregnancy: emerging role of relaxin. *Am J Physiol Regul Integr Comp Physiol*. 2011;301:R267–R275.
- Dschietzig T, Teichman S, Unemori E, Wood S, Boehmer J, Richter C, Baumann G, Stangl K. Intravenous recombinant human relaxin in compensated heart failure: a safety, tolerability, and pharmacodynamic trial. *J Card Fail*. 2009;15:182–190.
- Teichman SL, Unemori E, Teerlink JR, Cotter G, Metra M. Relaxin: review of biology and potential role in treating heart failure. *Curr Heart Fail Rep*. 2010;7:75–82.
- Ponikowski P, Mitrovic V, Ruda M, Fernandez A, Voors AA, Vishnevsky A, Cotter G, Milo O, Laessing U, Zhang Y, Dahlke M, Zymlinski R, Metra M. A randomized, double-blind, placebo-controlled, multicentre study to assess haemodynamic effects of serelaxin in patients with acute heart failure. *Eur Heart J*. 2014;35:431–441.
- Teerlink JR, Cotter G, Davison BA, Felker GM, Filippatos G, Greenberg BH, Ponikowski P, Unemori E, Voors AA, Adams KFJ, Dorobantu MI, Grinfeld LR, Jondeau G, Marmor A, Masip J, Pang PS, Werdan K, Teichman SL, Trapani A, Bush CA, Saini R, Schumacher C, Severin TM, Metra M; RELAXin in Acute Heart Failure (RELAX-AHF) Investigators. Serelaxin, recombinant human relaxin-2, for treatment of acute heart failure (RELAX-AHF): a randomised, placebo-controlled trial. *Lancet*. 2013;381:29–39.
- Vodstrcil LA, Tare M, Novak J, Dragomir N, Ramirez RJ, Wlodek ME, Conrad KP, Parry LJ. Relaxin mediates uterine artery compliance during pregnancy and increases uterine blood flow. *FASEB J*. 2012;26:4035–4044.
- Novak J, Parry LJ, Matthews JE, Kerchner LJ, Indovina K, Hanley-Yanez K, Doty KD, Debrah DO, Shroff SG, Conrad KP. Evidence for local relaxin ligand-receptor expression and function in arteries. *FASEB J*. 2006;20:2352–2362.
- Jelinic M, Leo CH, Post Uiterweer ED, Sadow SL, Gooi JH, Wlodek ME, Conrad KP, Parkington H, Tare M, Parry LJ. Localization of relaxin receptors in arteries and veins, and region-specific increases in compliance and bradykinin-mediated relaxation after in vivo serelaxin treatment. *FASEB J*. 2014;28:275–287.
- Jeyabalan A, Novak J, Doty KD, Matthews J, Fisher MC, Kerchner LJ, Conrad KP. Vascular matrix metalloproteinase-9 mediates the inhibition of myogenic reactivity in small arteries isolated from rats after short-term administration of relaxin. *Endocrinology*. 2007;148:189–198.
- McGuane JT, Danielson LA, Debrah JE, Rubin JP, Novak J, Conrad KP. Angiogenic growth factors are new and essential players in the sustained relaxin vasodilatory pathway in rodents and humans. *Hypertension*. 2011a;57:1151–1160.
- Jeyabalan A, Novak J, Danielson LA, Kerchner LJ, Opett SL, Conrad KP. Essential role for vascular gelatinase activity in relaxin-induced renal vasodilation, hyperfiltration, and reduced myogenic reactivity of small arteries. *Circ Res*. 2003;93:1249–1257.
- Danielson LA, Sherwood OD, Conrad KP. Relaxin is a potent renal vasodilator in conscious rats. *J Clin Invest*. 1999;103:525–533.
- Fisher C, MacLean M, Morecroft I, Seed A, Johnston F, Hillier C, McMurray J. Is the pregnancy hormone relaxin also a vasodilator peptide secreted by the heart? *Circulation*. 2002;106:292–295.
- McGuane JT, Debrah JE, Sautina L, Jarajapu YP, Novak J, Rubin JP, Grant MB, Segal M, Conrad KP. Relaxin induces rapid dilation of rodent small renal and human subcutaneous arteries via PI3 kinase and nitric oxide. *Endocrinology*. 2011b;152:2786–2796.
- Bani-Sacchi T, Bigazzi M, Bani D, Mannaioni PF, Masini E. Relaxin-induced increased coronary flow through stimulation of nitric oxide production. *Br J Pharmacol*. 1995;116:1589–1594.
- Edwards G, Feletou M, Weston AH. Endothelium-derived hyperpolarising factors and associated pathways: a synopsis. *Pflugers Arch*. 2010;459:863–879.
- Moncada S, Gryglewski R, Bunting S, Vane JR. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*. 1976;263:663–665.
- Danielson LA, Conrad KP. Time course and dose response of relaxin-mediated renal vasodilation, hyperfiltration, and changes in plasma osmolality in conscious rats. *J Appl Physiol*. 2003;95:1509–1514.
- van Drongelen J, van Koppen A, Pertijs J, Gooi JH, Parry LJ, Sweep FCGJ, Lotgering FK, Smits P, Spaanderman MEA. Impaired vascular responses to relaxin in diet-induced overweight female rats. *J Appl Physiol*. 2012;112:962–969.
- McPherson GA. Assessing vascular reactivity of arteries in the small vessel myograph. *Clin Exp Pharmacol Physiol*. 1992;19:815–825.
- Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res*. 1977;41:19–26.

23. Leo CH, Hart JL, Woodman OL. Impairment of both nitric oxide-mediated and EDHF-type relaxation in small mesenteric arteries from rats with streptozotocin-induced diabetes. *Br J Pharmacol*. 2011a;162:365–377.
24. Leo CH, Hart JL, Woodman OL. 3',4'-dihydroxyflavonol restores endothelium dependent relaxation in small mesenteric artery from rats with type 1 and type 2 diabetes. *Eur J Pharmacol*. 2011b;659:193–198.
25. Mazzuca MQ, Tare M, Parkington HC, Dragomir NM, Parry LJ, Wlodek ME. Uteroplacental insufficiency programmes vascular dysfunction in non-pregnant rats: compensatory adaptations in pregnancy. *J Physiol*. 2012;590:3375–3388.
26. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. *Trends Pharmacol Sci*. 2002;23:374–380.
27. Herrera EA, Verkerk MM, Derks JB, Giussani DA. Antioxidant treatment alters peripheral vascular dysfunction induced by postnatal glucocorticoid therapy in rats. *PLoS One*. 2010;5:e9250.
28. Wigg SJ, Tare M, Tonta MA, O'Brien RC, Meredith IT, Parkington HC. Comparison of effects of diabetes mellitus on an EDHF-dependent and an EDHF-independent artery. *Am J Physiol Heart Circ Physiol*. 2001;281:232–240.
29. Leo CH, Hart JL, Woodman OL. 3',4'-dihydroxyflavonol reduces superoxide and improves nitric oxide function in diabetic rat mesenteric arteries. *PLoS One*. 2011c;6:e20813–e20823.
30. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288:373–376.
31. Palmer RM, Ashton DS, Moncada S. Vascular endothelium cells synthesize nitric oxide from L-arginine. *Nature*. 1988;333:664–666.
32. Conrad KP, Shroff SG. Effects of relaxin on arterial dilation, remodeling, and mechanical properties. *Curr Hypertens Rep*. 2011;13:409–420.
33. Dschietzig T, Brecht A, Bartsch C, Baumann G, Stangl K, Alexiou K. Relaxin improves TNF- α -induced endothelial dysfunction: the role of glucocorticoid receptor and phosphatidylinositol 3-kinase signalling. *Cardiovasc Res*. 2012;95:97–107.
34. Conrad KP. Unveiling the vasodilatory actions and mechanisms of relaxin. *Hypertension*. 2010;56:2–9.
35. Dudzinski DM, Michel T. Life history of eNOS: partners and pathways. *Cardiovasc Res*. 2007;75:247–260.
36. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999;399:597–601.
37. Novak J, Ramirez RJ, Gandle RE, Sherwood OD, Conrad KP. Myogenic reactivity is reduced in small renal arteries isolated from relaxin-treated rats. *Am J Physiol Regul Integr Comp Physiol*. 2002;283:R349–R355.
38. Sandow SL, Tare M. C-type natriuretic peptide: a new endothelium-derived hyperpolarizing factor? *Trends Pharmacol Sci*. 2007;28:61–67.
39. Absi M, Burnham MP, Weston AH, Harno E, Rogers M, Edward G. Effects of methyl beta-cyclodextrin on EDHF responses in pig and rat arteries; association between SK_{Ca} channels and caveolin-rich domains. *Br J Pharmacol*. 2007;151:332–340.
40. Dora KA, Gallagher NT, McNeish A, Garland CJ. Modulation of endothelial cell KCa3.1 channels during endothelium-derived hyperpolarizing factor signalling in mesenteric resistance arteries. *Circ Res*. 2008;102:1247–1255.
41. Sandow SL, Neylon CB, Chen M, Garland CJ. Spatial separation of endothelial small- and intermediate-conductance calcium-activated potassium channels (K_{Ca}) and connexins: possible relationship to vasodilator function? *J Anat*. 2006;209:689–698.
42. Sandow SL, Tare M, Coleman HA, Hill CE, Parkington HC. Involvement of myoendothelial gap junctions in the actions of endothelium-derived hyperpolarizing factor. *Circ Res*. 2002;90:1108–1113.
43. Chan SL, Cipolla MJ. Relaxin causes selective outward remodeling of brain parenchymal arterioles via activation of peroxisome proliferator-activated receptor- γ . *FASEB J*. 2011;25:3229–3239.
44. Chan SL, Sweet JG, Cipolla MJ. Treatment for cerebral small vessel disease: effect of relaxin on the function and structure of cerebral parenchymal arterioles during hypertension. *FASEB J*. 2013;27:3917–3927.
45. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev*. 1999;79:1193–1226.
46. Unemori EN, Lewis M, Grove BH, Deshpande U. Relaxin induces specific alterations in gene expression in the human endometrium. In: Tregear GW, Ivell R, Bathgate RA, Wade JD, eds. *Relaxin*. The Netherlands: Kluwer Academic Publishers; 2000:65–72.
47. Collino M, Rogazzo M, Pini A, Benetti E, Rosa AC, Chiazza F, Fantozzi R, Bani D, Masini E. Acute treatment with relaxin protects the kidney against ischaemia/reperfusion injury. *J Cell Mol Med*. 2013;17:1494–1505.
48. Gooi JH, Richardson M, Jelinic M, Girling J, Wlodek ME, Tare M, Parry LJ. Decreased uterine and mesenteric artery compliance in pregnant mutant mice. *Reprod Sci*. 2013;20(suppl):O–O40.
49. Gooi JH, Richardson ML, Jelinic M, Girling JE, Wlodek ME, Tare M, Parry LJ. Enhanced uterine artery stiffness in aged pregnant relaxin mutant mice is reversed with exogenous relaxin treatment. *Biol Reprod*. 2013;89:1–11.