ONLINE SUPPLEMENT

ANGIOGENIC GROWTH FACTORS ARE NEW AND ESSENTIAL PLAYERS IN THE SUSTAINED RELAXIN VASODILATORY PATHWAY IN RODENTS AND HUMANS

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Short title: VEGF and PGF Mediate Relaxin-Induced Vasodilation

Expanded Methods

Animals

Female Long-Evans rats and C57B6/J mice (both 12-14 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN) and housed under standard conditions (12:12 light/dark cycle) with access to PROLAB RMH 2000 feed containing either 0.32% or 0.48% sodium (PME Feeds Inc., St. Louis, MO) and water ad libitum. Mice and rats not used for renal function studies were euthanized with pentobarbital (60 mg/kg i.p.) followed by thoracotomy, and small renal arteries (SRA) were harvested for study of myogenic constriction *in vitro* as described below. All procedures involving animals were approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh or University of Florida.

Human arteries

Subcutaneous fat was obtained from patients undergoing elective surgery at the Magee Womens Hospital, University of Pittsburgh under the approval of the University of Pittsburgh Institutional Review Board. A retrospective analysis of the available patient data indicates that the tissues were predominantly from females aged 20 – 60 years; only two patients were male. The tissue sources were pannus (n=12), breast (n=5), buttock (n=1), upper leg (n=1) and eyelid (n=1). Small arteries were carefully dissected from the tissue and prepared for *in vitro* analysis of myogenic reactivity as described below.

Myogenic behavior of isolated arteries

The effect of relaxin on myogenic constriction was assessed in isolated small renal and subcutaneous arteries from mice and rats, and humans, respectively, as previously described (1-6). This bioassay exploits the well-known phenomenon of arterial constriction in response to increases in intraluminal pressure (myogenic constriction), an effect that may be attenuated by vasodilators. This is distinct from myogenic dilation, which may be observed by *decreasing* intraluminal pressure, whereupon arteries *dilate* in response to this stimulus, but this aspect of arterial behavior was not studied.

Briefly, arterial segments were transferred to a dual chamber isobaric arteriograph (Living Systems Instruments, Burlington, VT) containing 3mL HEPES-buffered PSS (pH 7.4) maintained at 37°C. Arteries were mounted and secured with ties on the proximal microcannula (connected to a peristaltic pump and pressure servo controller), and residual blood was flushed under gentle pressure (≤5 mmHg), before being mounted on the distal microcannula with ties. Arteries were pressurized to 60 mmHg and the buffer was replaced. Recombinant human H2 relaxin (rhRLX, 30 ng/ml; Corthera, San Mateo, CA) or vehicle (20 mmol/L sodium acetate, pH 5 diluted identically as the stock rhRLX) was then added to the bath, and arteries were incubated for 3 hr; the buffer was changed every 30 min. Some arteries were incubated with relaxin-3 or Insl-3 (other members of the relaxin- and insulin-like peptide family; (7) instead of rhRLX. After 3 hr, various inhibitors/neutralizing antibodies or their vehicles/control antibodies were added to the arterial preparation (in the continuing presence of rhRLX or its vehicle). In the case of angiogenic growth factor blockade, this procedure was also reversed (i.e., incubation with SU5416/neutralizing antibodies or dilute DMSO/control antibodies for 30 min, followed by

treatment with rhRLX or vehicle in the continuing presence of the inhibitor/neutralizing antibody or vehicle/control antibody for 3 hr). To economize reagents, MMP-2 and -9, as well as VEGF- and PGF-neutralizing and control antibodies were instilled intraluminally.

A conditioning stretch (60 – 100 mmHg) was performed over a period of approximately one minute after the first and penultimate buffer changes. After the final buffer change, arteries were constricted to approximately 80% of initial internal diameter using phenylephrine (Sigma-Aldrich, St. Louis, MO) (1,6). The only exception to this was for mouse SRA, which naturally possess sufficient intrinsic tone to permit observation of attenuated myogenic constriction (J. Novak, unpublished observations). Internal diameter was assessed using a model 1602-E filar with SM-2 processor (Lasico, Los Angeles, CA) mounted on a TS100 inverted microscope (Nikon, Melville, NY). Myogenic constriction was determined by measuring internal diameter at 60 mmHg, then increasing intraluminal pressure by 20 mmHg in stepwise fashion and allowing the vessel to stabilize (4 – 6 min) before measuring internal diameter at 80 mmHg. Intraluminal pressure was then returned to 60 mmHg and, after another 4 – 6 min stabilization period, the process was repeated. A total of three replicates were performed and the values were averaged.

Despite originating from the same anatomic location within the relevant vascular bed, the initial diameter of the arteries used for these studies is inherently variable as illustrated in **Supplemental Figure S1**. In order to account for this, the results are expressed as % change in diameter from baseline at 60 mmHg (i.e., no change in diameter indicates relatively robust myogenic constriction, whereas an increase in diameter reflects attenuation of myogenic constriction). Studies were conducted such that individual arteries from the same animal were subjected to both "control" and "experimental" conditions (i.e., in a paired fashion). Therefore, n represents the sample size in terms of both arteries and animals.

Some arteries were also studied under passive conditions after the completion of an experiment as described above. Briefly, the HEPES-buffered PSS was washed out and replaced with Ca^{2+}-free buffer containing 100 μmol/L papaverine and EGTA (both Sigma-Aldrich, St Louis, MO). After 15 minutes, internal diameter at 60 mmHg was measured before increasing intraluminal pressure to 80 mmHg in stepwise fashion and again assessing internal diameter.

The inhibitors used were 100 μmol/L N^G-monomethyl-L-arginine acetate (L-NMMA, a competitive inhibitor of NOS; Sigma-Aldrich) (8), 10 μmol/L RES-701-1 (ET_B receptor antagonist; Kyowa Hakko Kogyo, Japan) (1,6,9), 1 μmol/L GM6001 (general MMP inhibitor; Chemicon Millipore, Billerica, MA) (2), 1 μmol/L SU5416 (VEGF receptor tyrosine kinase inhibitor; Sigma-Aldrich) (10), as well as MMP-2 and -9, VEGF and PGF neutralizing antibodies at appropriate concentrations. Details of the neutralizing and control antibodies used are presented in **Supplemental Table S1**.

Renal function studies in conscious rats

Surgical procedures. Rats were chronically instrumented as described previously for assessment of renal function (11,12). Renal function studies were conducted in rodent experimental conditioning units (ECU; Braintree Scientific Co., Braintree, MA), which permits accurate timed urine collection and blood sampling via bladder and arterial catheters, respectively. Prior to surgery, rats were acclimated to the ECU by placing them in the ECU for 1-3 hr per day on at least five different days.

Briefly, the surgical protocol consisted of anaesthesia induction by 5% isoflurane and maintenance thereafter with 1.5% isoflurane followed by implantation of Tygon catheters into the abdominal aorta and inferior vena cava via the left femoral artery and vein, respectively. The vascular catheters were tunneled subcutaneously and exteriorized between the scapulae. A third catheter consisting of a silastic-coated stainless steel cannula was sewn into the urinary bladder with a purse-string suture and exteriorized through the ventral abdominal wall. This catheter was then plugged, which allowed the animal to urinate normally while not under study. For analgesia, buprenorphine (0.05 mg/kg s.c) was administered after surgery.

Experimental Protocol. The experimental protocol followed the timeline illustrated in **Figure 1**. Seven days after surgery, baseline measurements of glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and mean arterial pressure (MAP) were made. SU5416 (1,3-Dihydro-3-[(3,5-dimethyl-1H-pyrrol-2-yl)methylene]-2H-indol-2-one) dissolved in sterile, endotoxin-free DMSO or the DMSO vehicle alone (both 150 µl and from Sigma-Aldrich) was then injected s.c (20 mg/kg/d), which was repeated daily for the duration of the study. This dose of SU5416 used *in vivo* was based on extensive literature (13-18). Approximately 24 hr after the first SU5416 injection, a 7-day Alzet osmotic pump (model 2001; Durect Corporation, Cupertino, CA) containing rhRLX or sodium acetate vehicle (20 mmol/L, pH 5.2) was implanted s.c in the back under isoflurane anaesthesia, a procedure lasting ~10 min. The infusion rate of rhRLX was 4 µg/h, which yields circulating concentrations of the hormone similar to those observed in midterm pregnant rats (when the gestational increases in ERPF and GFR peak in this species) (11,19). GFR, ERPF and MAP were then assessed 4-6 hr and 3-5 days after the start of the rhRLX or vehicle infusion. After the final measurement of renal function, rats were anaesthetized using isoflurane, and a laparotomy was performed. Trunk blood was collected from the abdominal aorta. Both kidneys were removed and placed into saline on ice. The renal arteries were collected by bisecting each kidney longitudinally, and removing the medulla and papilla, in order to expose the underlying interlobar vessels. Three to five arteries were dissected from each kidney half, pooled and snap frozen in liquid nitrogen for analysis of gelatinase activity.

Assessment of renal function and MAP. To measure GFR, ERPF and MAP, rats were first placed in the ECU (11,12). The femoral artery catheter was then connected to a Research BP Transducer and Universal Oscillograph (Harvard Apparatus, Holliston, MA) for continuous measurement of MAP. Next, a bolus of inulin (IN; 0.2 ml of a 20% stock solution/100 g body weight; Sigma-Aldrich) and para-aminohippurate (PAH; 0.1 ml of a 20% stock solution/100 g body weight; Merck & Co., Whitehouse Station, NJ) were infused over 1 min via the femoral venous catheter, followed by constant infusion in Ringer's solution at 0.4 and 0.1 mg/min/100 g body weight for measurement of GFR and ERPF, respectively. The flow rate was 19 µl/min, delivered by a model 200 syringe pump (KD Scientific, Boston, MA, USA). The obturator was then removed from the bladder catheter and a short piece of silastic tubing was attached to facilitate urine collection. After equilibration for 1 hr, three urine collections of 30 min each with midpoint blood collections of 200 µl each were obtained. Plasma was obtained by centrifugation and erythrocytes were resuspended in Ringer's solution and returned to the animal.

Analytical Techniques. Plasma and urine IN and PAH concentrations were analyzed by standard techniques as reported previously (11,12). Plasma rhRLX concentrations were measured in duplicate using a commercially available ELISA that has been validated for rat plasma (R&D Systems, Minneapolis, MN). The assay yielded a standard curve with R^2=0.99, and the minimum detectable dose (MDD) was 9.2 pg/ml. The intra-assay precision (average CV of the unknowns) was 1.8 ± 0.6%. Plasma H2 relaxin was below the MDD in vehicle-treated rats.

Arterial gelatinase activity

Arterial gelatinase activity was measured by gelatin zymography as previously described with minor modifications (2,4,20). Briefly, SRA harvested after the last renal function and MAP measurements were placed into a capsule containing a steel ball bearing, which was cooled in liquid nitrogen, and pulverized using a WIG-L-BUG amalgamator (Dentsply, York, PA). Five volumes of homogenization buffer containing 10 mM Tris pH 6.8, 7 M urea, 10% glycerol and 1% SDS supplemented with 10 µl Protease Inhibitor Cocktail Set III (EMD Chemicals, Gibbstown, NJ) per ml of buffer was added to the tissue, which was then homogenized in the WIG-L-BUG. The homogenate was sonicated for 1-2 s using a Misonix 2000 on setting 4 (Qsonica, Newtown, CT) and centrifuged at 15,000 × g for 10 min at 4°C. The supernatant was subsequently transferred to a new tube, and protein concentration was determined in triplicate (at two dilutions when possible) using the *DC* Protein Assay (Bio-Rad, Hercules, CA). The average protein concentration was calculated and samples were diluted in additional homogenization buffer as needed.

Five µg protein was combined with an equal volume of 2× Novex® Tris-Glycine SDS Sample Buffer and electrophoresed on Novex® 10% Zymogram (gelatin-containing) Gels for approximately 2 hr at 100 V. Gels were then incubated in Novex® Zymogram Renaturing Buffer followed by Novex® Zymogram Developing Buffer (all from Invitrogen, Carlsbad, CA), both at room temperature with gentle agitation for 30 min. The buffer was then replaced with fresh Developing Buffer and gels were incubated at 37°C for approximately 18 hr. Staining and destaining of gels was then performed with gentle agitation at room temperature, using fresh 0.5% Brilliant Blue R250 (Fisher Scientific, Pittsburgh, PA) in 30% methanol and 10% acetic acid for 70 min, followed by two 15 min rinses in 30% methanol/10% acetic acid. Further destaining took place in 1% Triton X-100 for 1 hr, after which gels were imaged using the AlphaEase FluorChem imaging system (Alpha Innotech, San Leandro, CA). Bands of interest were delineated and quantified using Scion Image software (Frederick, MD).

Statistical analysis

Parameters of renal function and MAP were analyzed by two-way repeated measures ANOVA. If significant main effects or interactions were obtained, then individual group means were contrasted by Fisher's least significant difference test (SAS 9.2, Cary, NC). Isolated artery data (myogenic reactivity) were analyzed by the non-parametric Wilcoxon signed-ranks test for related samples or (in the case of the occasional failure of an artery during an experiment) the Krusal-Wallis or Mann-Whitney U test for independent samples (PASW Statistics 18.0; IBM Corp., Somers, NY). For zymography, a one sample t-test was applied. $P<0.05$ was considered statistically significant. Occasional deviations in the statistical analyses from those described above are noted in Figure and Table Legends, or Results.

Results and Discussion of Supplemental Figures 1 & 2

We previously reported that myogenic constriction is attenuated in small renal and mesenteric arteries isolated from relaxin-treated nonpregnant rats relative to small arteries from vehicle-treated nonpregnant rats over a broad range of intraluminal pressures (1). We subsequently developed and validated an abbreviated protocol as a more convenient and time-saving approach in which myogenic constriction is measured in response to a step increase in intraluminal pressure from 60 to 80 mmHg (1,6). This abbreviated protocol also allows for multiple measurements of myogenic constriction in each artery (in our case 3 replicates), thus improving precision (see **Expanded Methods** above). Further, this abbreviated protocol minimizes the possibility of deterioration of the preparation that may occur while testing an entire range of pressures *after* the 3 hr incubation with relaxin or vehicle *in vitro* as performed in the current work.

The attenuation of myogenic constriction in small renal arteries isolated from nonpregnant rats treated with relaxin has been a highly reproducible bioassay (1,2,4,21), current work), and mimics the attenuation of myogenic constriction of small renal arteries from midterm pregnant rats (2,6). Moreover, in midterm pregnant rats treated with relaxin neutralizing antibodies or subjected to ovariectomy (thus removing the source of circulating relaxin), the normal pregnancy phenotype of attenuated myogenic constriction in small renal arteries is restored to the nonpregnant phenotype of robust myogenic constriction (3).

Finally, the attenuation of myogenic constriction in small renal arteries from relaxin-treated nonpregnant rats or from pregnant rats is restored to the nonpregnant phenotype by endothelial removal (1), nitric oxide synthase (NOS) blockade with L-NAME or L-NMMA (1,6), the ET_B receptor antagonist RES-701-1 or the mixed $ET_A/_B$ antagonist SB209670, but not the ET_A antagonist, BQ123 (1,6); the general MMP antagonist, GM6001 (2), a specific MMP-2 antagonist, cyclic CTT (2) and gelatinase-neutralizing antibodies (2,4). Blockade of NOS, the ET_B receptor and MMPs also inhibit relaxin- and/or pregnancy-induced renal vasodilation and hyperfiltration in conscious rats (2,6,12,22-24). Thus, there is excellent consistency between *in vivo* studies and the myogenic constriction bioassay with respect to molecular mechanisms. In **Supplemental Figures S1** & **S2**, we present additional analysis supporting the validity of this bioassay as a tool with which to further interrogate the vasodilatory actions of relaxin *in vitro*, and justifying presentation of the data in the main manuscript as % change in diameter from baseline at 60 mmHg ($[D_{80}-D_{60}]/D_{60}\times100$ %). These figures were compiled from the raw data summarized in **Figures 2** & **3**, and **Table 1**.

Supplemental Figure S1 depicts the internal diameters of all the small arteries studied in this work at 60 and 80 mmHg (average of three replicates each). Rat and mouse small renal, and human subcutaneous arteries were treated *in vitro* with 30 ng/ml rhRLX + pharmacologic or immunologic vehicles/controls (**A**) or 30 ng/ml rhRLX + pharmacologic or immunologic inhibitors (**B**). Many of the same arteries were subsequently incubated with papaverine & EGTA (both 100 μmol/L) in Ca^{2+}-free (passive) buffer and myogenic constriction again measured (**C**). In **A** & **C**, there is a highly significant, albeit small increase in the mean internal diameter at 80 compared to 60 mmHg (both $P<0.0001$), which is not observed in **B** (P=NS). Significance is reached in **A** & **C** despite the large scatter in internal diameters at 60 mmHg (also see **Supplemental Figure S2**) because of the *consistent increase* in internal diameter from 60 to 80 mmHg among the arteries. Thus, myogenic constriction was attenuated in the presence of rhRLX

+ pharmacologic or immunologic vehicles/controls (**A**), and in passive buffer (**C**), relative to rhRLX + pharmacologic or immunologic inhibitors (**B**). This finding is also supported by the mean change in internal diameter from 60 to 80 mmHg as shown below each panel, which was significantly greater in the presence of rhRLX + pharmacologic or immunologic vehicles/controls and passive buffer relative to rhRLX + pharmacologic or immunologic inhibitors ($P<0.01$).

In **Supplemental Figure S2,** myogenic constriction of small arteries is presented as a function of initial diameter (i.e., measured before conducting the experiment). The change in diameter is expressed both as the absolute difference in diameters at 60 and 80 mmHg in microns (**A** & **C**), and as percent change in diameter between 60 and 80 mmHg relative to the 60mmHg baseline (**B** & **D**). A mixed-effect model was fit for this analysis using initial diameter as a covariate because of the scatter in arterial diameter as described above.

First, there is no significant effect of the initial diameter on the *absolute change* in diameter from 60 to 80 mmHg in the case of rhRLX + pharmacologic or immunologic inhibitors (**open diamonds, A**). However, there is a significant, albeit small, positive effect of initial diameter in the case of rhRLX + pharmacologic or immunologic vehicles/controls (**black squares**, **A**), and passive conditions (**shaded diamonds, C**; $P<0.0001$ and $P<0.05$ respectively). The slopes of the linear regressions indicate that for every 1.0 μm increase in initial diameter there is a 0.04 and 0.02 μm increase in the change in diameter from 60 to 80 mmHg, respectively.

Second, and in contrast, initial diameter does not significantly affect the *percent change* in diameter from 60 to 80 mmHg (**B** & **D**). Therefore, expressing myogenic constriction as percent change normalizes for the interaction effect of initial diameter in arteries treated with rhRLX + pharmacologic or immunologic vehicles/controls (i.e., in which myogenic constriction is attenuated) and under passive conditions.

Third, regardless of whether the data are expressed as absolute change or percent change, there is a significant main effect of treatment between arteries treated with rhRLX + pharmacologic or immunologic vehicles/controls (**black squares**) and arteries treated with rhRLX + pharmacologic or immunologic inhibitors (**open diamonds,** $P<0.0001$ both **A** & **B**). Thus, treatment with rhRLX + pharmacologic or immunologic vehicles/controls attenuates myogenic constriction relative to rhRLX + pharmacologic or immunologic inhibitors, in which there is robust myogenic constriction.

In summary, incubation of small arteries with rhRLX *in vitro* attenuates myogenic constriction, a subtle but highly significant (both physiologically and statistically) effect. Expression of the data as percent change ($[D_{80}-D_{60}]/D_{60} \times 100\%$) normalizes for variation in initial diameter among arteries, which can be considerable (*supra vide*). The positive relationship between initial diameter and absolute change in diameter in arteries treated with rhRLX + pharmacologic or immunologic vehicles/controls or with papaverine & EGTA in Ca^{2+}-free buffer provides further rationale for expressing the data as percent change, because it accounts for this effect. It also visually simplifies the presentation of a biologically complex phenomenon for the reader (e.g., **Figures 2** & **3**).

It is also worth pointing out that the impact of initial diameter on the absolute change in the presence of rhRLX + pharmacologic or immunologic vehicles/controls, but not rhRLX + pharmacologic or immunologic inhibitors, is somewhat moot, because there were actually no significant differences in the initial diameters of rat, mouse or human arteries between these two treatment groups (see legends to **Figures 2** & **3**).

A final point of clarification is that myogenic constriction in rhRLX-treated arteries and under passive conditions is not directly comparable, strictly speaking. The presentation of the two data sets in **Supplemental Figures S1** & **S2** serves only to illustrate that the attenuation of myogenic constriction by rhRLX is *similar* to that observed in the presence of papaverine & EGTA in Ca^{2+}-free buffer.

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Neutralizing antibody	Control antibody	Concentration	Cross Reactivity #
anti-mouse VEGF (R&D Systems Cat# AF-493-NA)	goat IgG	1 µg/ml *	0 - <5% rhPGF; 0% rmPGF-2
anti-human VEGF (R&D Systems Cat# AF-293-NA)	goat IgG	1 µg/ml *	0% rhPGF
anti-rat VEGF (R&D Systems Cat# AF-564)	goat IgG	3 µg/ml *	0% rhPGF & rmPGF-2
anti-mouse and rat PGF-2 (R&D Systems Cat# MAB465) †	rat IgG_{2A}	1.0 and 0.1 µg/ml *	0% rhVEGF and rhPGF
anti-human PGF (R&D Systems Cat# MAB264)	mouse IgG_1	10 µg/ml ‡	0% rhVEGF
Anti-human rat and mouse MMP-2 (Chemicon Cat# MAB13405)	mouse IgG_{1k}	3 µg/ml §¶	
Anti-rat and mouse MMP-9 (Lab Vision Cat# MS-819-p1ABX)	mouse IgG_{1k}	10 µg/ml ¶	

Supplemental Table S1. Sources and characteristics of neutralizing antibodies used in vitro.

* Concentration based on manufacturer's assays of neutralization of VEGF stimulated 3H-thymidine incorporation into human umbilical vein endothelial cells.

† Rat and mouse PGF share 92% homology.

‡ Concentration adapted from (25).

§ Concentration as used previously (26).

¶ Concentration as used previously (27).

Cross-reactivities tested by R&D Systems on ELISA, reducing and/or non-reducing Western blot.

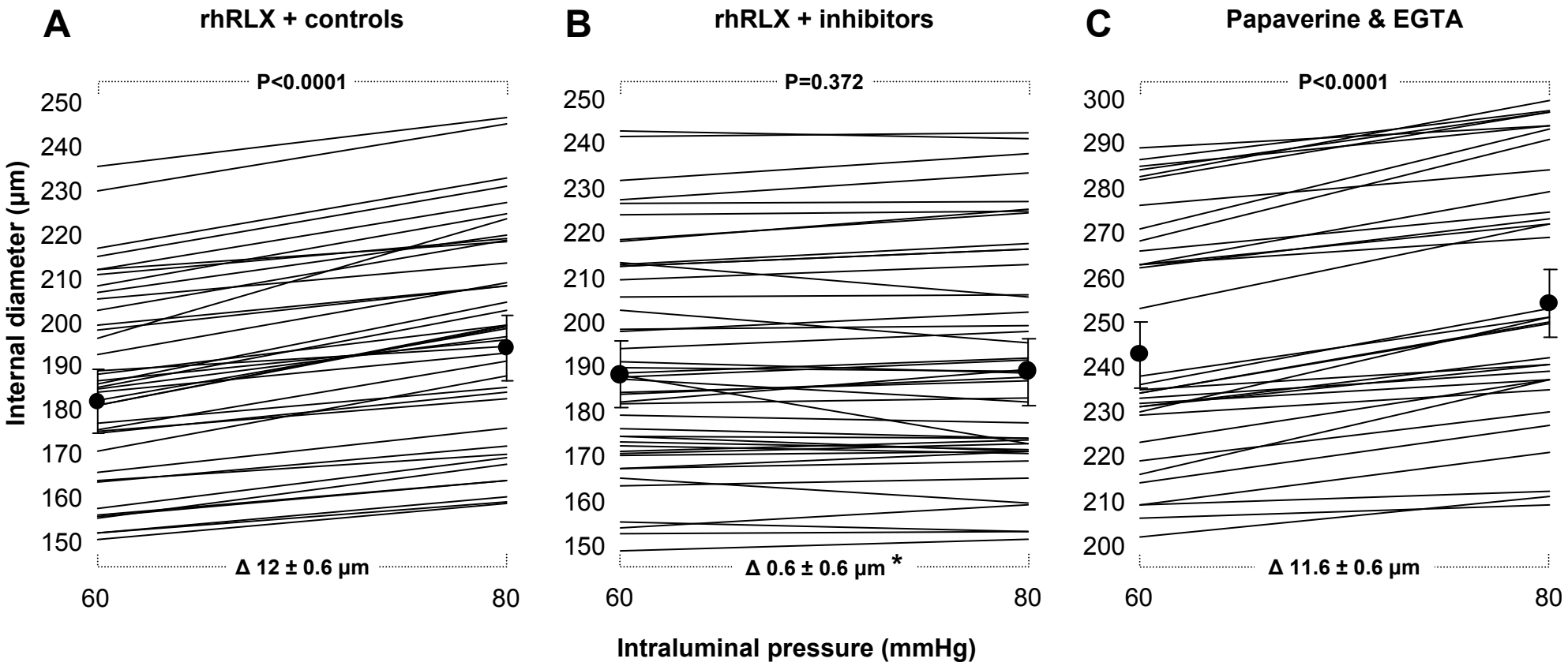
Rat small renal arteries

	Vehicle for rhRLX + vehicle or control Ab	Vehicle for rhRLX + inhibitor or neutralizing Ab
GM6001	1.6 ± 0.6 (n=3 rats)	1.4 ± 0.6 (n=3 rats)
RES-701-1	0.0 ± 1.0 (n=3 rats)	1.1 ± 1.5 (n=3 rats)
L-NMMA	2.3 ± 0.1 (n=2 rats)	0.6 ± 1.4 (n=3 rats)
VEGF Ab	1.5 ± 2.1 (n=4 rats)	-0.4 ± 0.7 (n=4 rats)
PlGF Ab	1.1 ± 1.3 (n=3 rats)	1.3 ± 1.0 (n=3 rats)

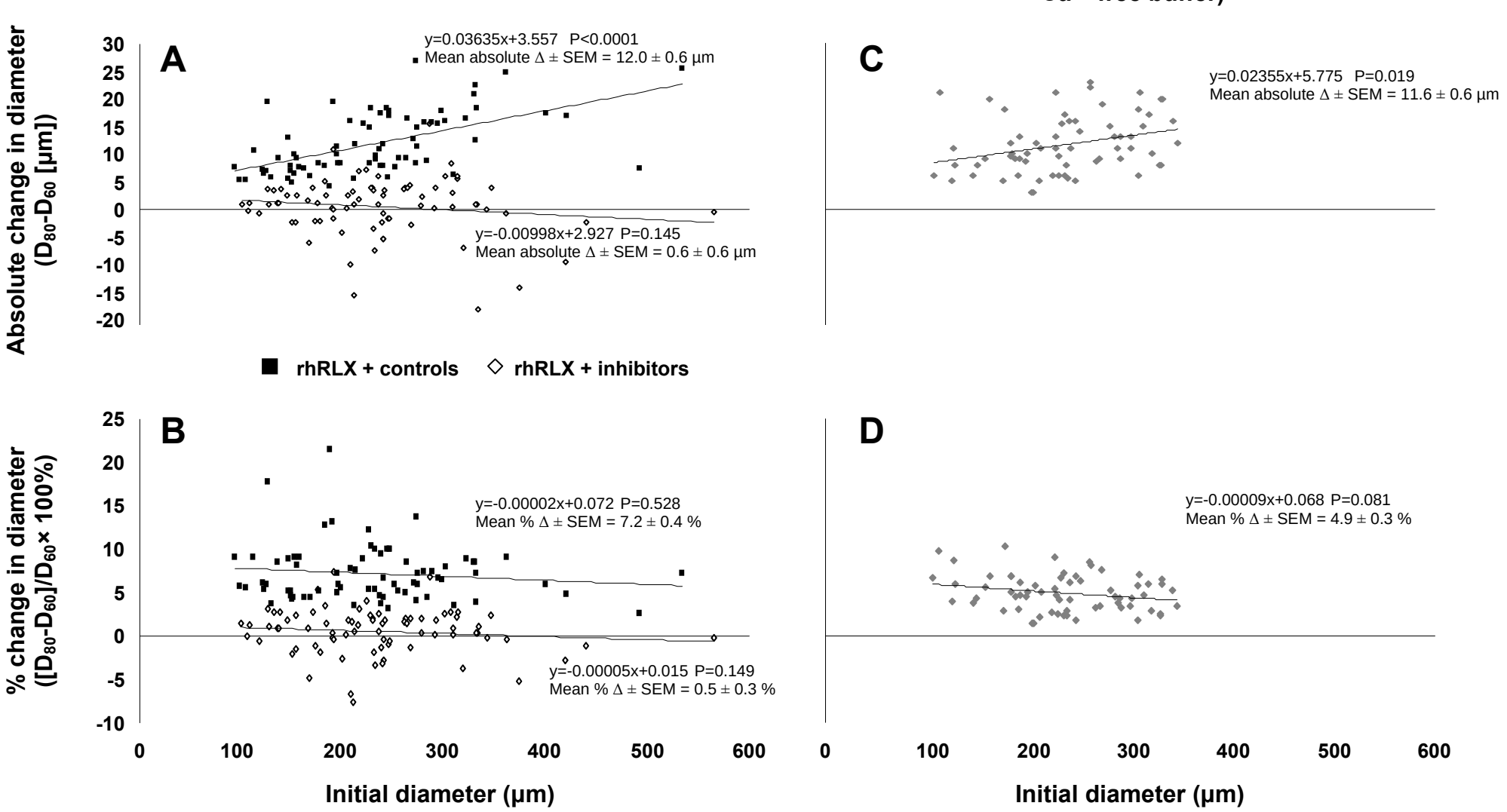
Human subcutaneous arteries

	Vehicle for rhRLX + vehicle	Vehicle for rhRLX + inhibitor
L-NMMA	-0.8 ± 0.6 (n=5 patients)	-0.7 ± 0.6 (n=5 patients)

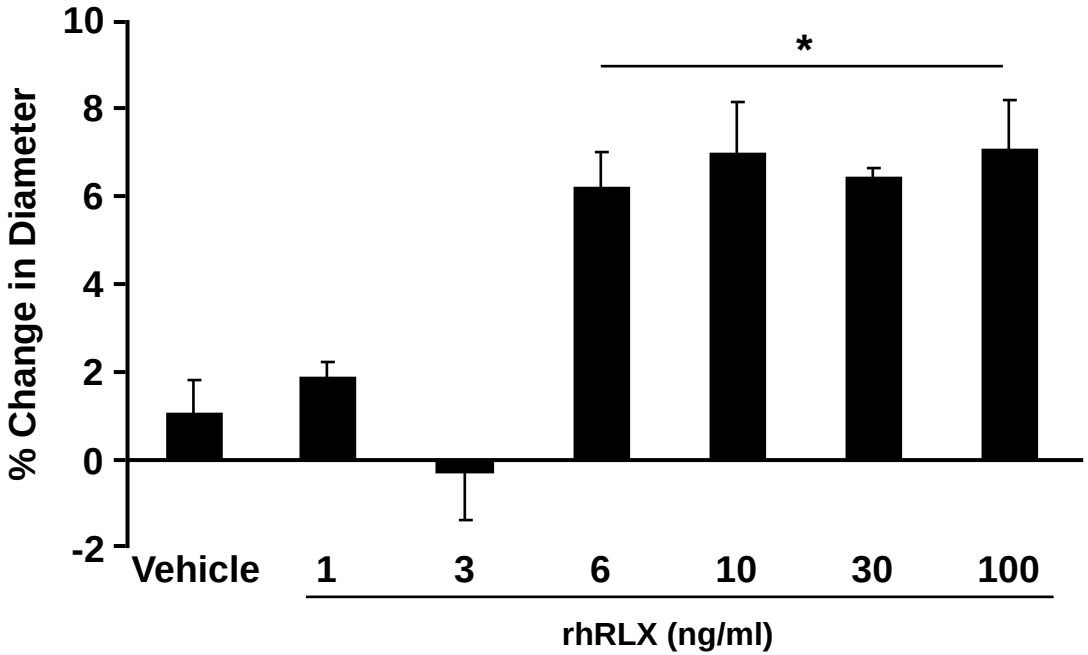
Supplemental Table S2. Control experiments. Arteries were isolated and incubated with the vehicle for rhRLX (a sodium acetate solution) for 3 hr *in vitro*. The 20 mM stock solution (pH 5) was diluted to the same extent as rhRLX in HEPES-buffered PSS (pH 7.4) for use. Inhibitors/neutralizing antibodies (Ab) or their respective vehicles/control Ab were then added to the bath for an additional 30 min (in the continuing presence of sodium acetate vehicle for rhRLX), after which myogenic reactivity was measured. Myogenic reactivity is expressed as % change in internal diameter from 60 to 80 mmHg (mean ± SEM). See **Expanded Methods** for details. There were no significance differences between vehicle/ control Ab and inhibitor/neutralizing Ab treatments.



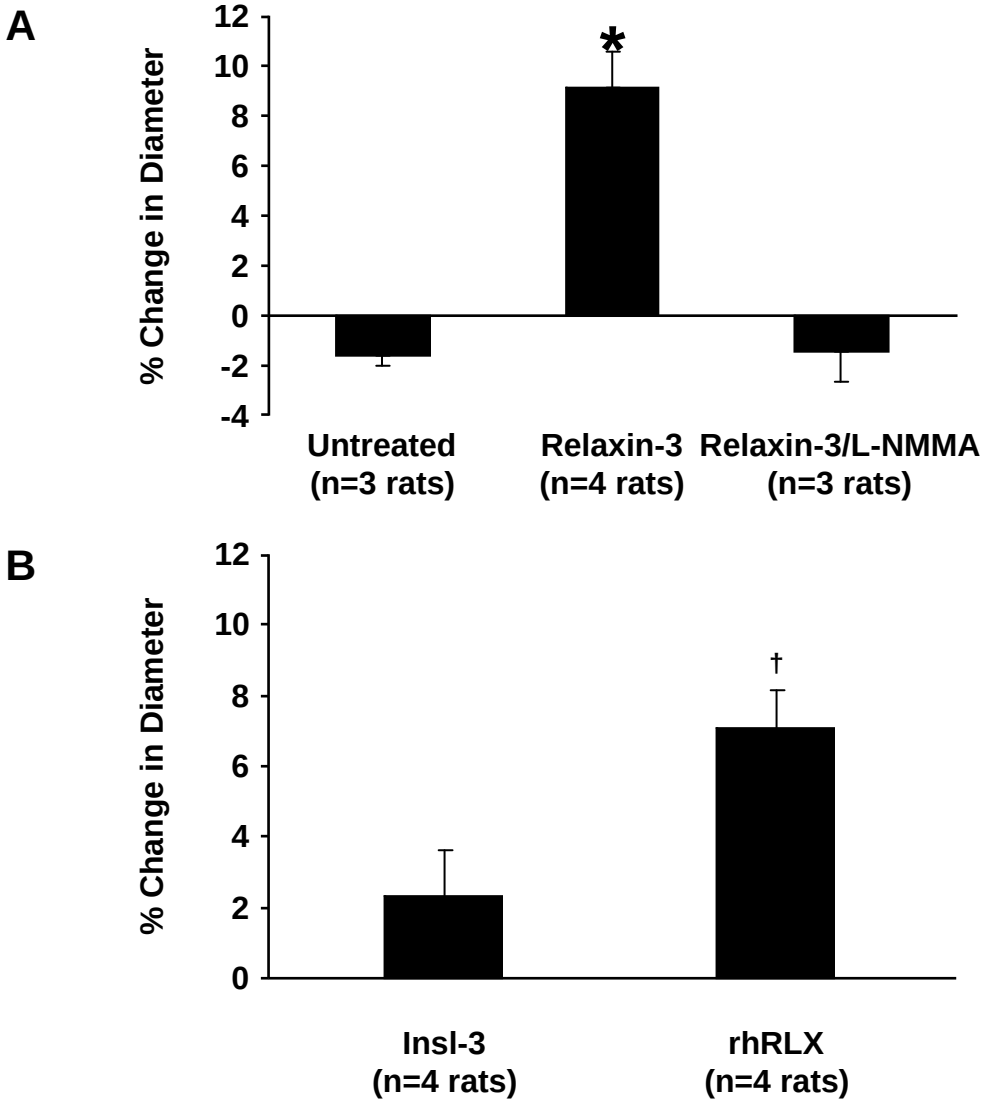
Supplemental Figure S1. Myogenic reactivity of isolated small arteries (absolute internal diameters at 60 & 80 mmHg). Rodent small renal and human subcutaneous arteries were treated with (**A**) 30 ng/ml rhRLX + pharmacologic or immunologic controls, or (**B**) 30 ng/ml rhRLX + pharmacologic or immunologic inhibitors for 3 hr *in vitro*. (**C**) Many of the same arteries were subsequently incubated in papaverine & EGTA (both 100 µmol/L) in Ca^{2+}-free (passive) buffer for 15 minutes and myogenic reactivity was again measured. Depicted in each panel is the change in internal diameter from 60 and 80 mmHg for each artery (average of 3 replicates per artery; see **Expanded Methods**) and the mean ± SEM internal diameter at 60 and 80 mmHg (**black circles**). For each panel, the internal diameters at 60 and 80 mmHg were analysed by Wilcoxon Signed Ranks Test. Mean difference from 60 to 80 mmHg for **A**, **B** & **C** is shown below each panel. * P< 0.01 vs **A** (Δ 12 $\pm$ 0.6 µm) and **C** (Δ 11.6 $\pm$ 0.6 µm) by one way ANOVA and LSD test. For clarity of presentation, only arteries with internal diameters between 150 - 250 µm for **A** & **B**, and 250 - 350 µm for **C** are shown. The majority of arteries fell within these ranges, but the few above and below showed identical behavior and were included in the analysis. Internal diameter at 60 mmHg was not significantly different between arteries in **A** & **B** (P = NS by unpaired t-test).



Supplemental Figure S2. Myogenic reactivity of isolated small arteries as a function of initial internal diameter: absolute change in internal diameter at 60 and 80 mmHg (A & C), and percent change in internal diameter (B & D). In **A** & **B**: **black squares**, arteries treated with 30 ng/ml rhRLX plus pharmacologic or immunologic controls (rhRLX + controls); **open diamonds**, arteries treated with 30 ng/ml rhRLX plus pharmacologic or immunologic inhibitors (rhRLX + inhibitors). In **C** & **D**: **shaded diamonds**, many arteries depicted in **A** & **B** were subsequently incubated with papaverine & EGTA (both 100 µmol/L) in Ca^{2+}-free (passive) buffer for 15 minutes and myogenic reactivity was again measured. Absolute and % change in internal diameters for each artery represent the average of 3 replicates per artery (see **Expanded Methods**). Initial internal diameter of all arteries treated with rhRLX + controls or rhRLX + inhibitors was not significantly different (230.5 ± 10.2 µm vs 235.5 ± 9.4 µm, respectively; P=NS by unpaired t-test). Mixed model analyses revealed a significant main effect of treatment whether the data are expressed as absolute (**A**) or % change (**B**) (both P<0.0001; rhRLX + controls vs rhRLX + inhibitors). There is also a significant interaction between initial internal diameter and treatment in **A** (rhRLX + control group only) & **C**; this effect disappears when the data are expressed as % change (**B** & **D**; see panels for individual P values). Finally, and in accordance with the previous, the slope of the rhRLX + inhibitor group (**A**) is significantly different from the rhRLX + control group (**A**) as well as the passive group (**C**) (P<0.0001 & P<0.005, respectively), whereas the slopes of the rhRLX + control (**A**) and passive groups (**C**) are not significantly different (P=0.275). Again, these slope differences disappear upon conversion to % change (**B** & **D**; all P=NS).



Supplemental Figure S3. Recombinant human relaxin (rhRLX) dose response (myogenic reactivity) in rat small renal arteries. Arteries were isolated and incubated for 3 hr in vitro with rhRLX at various concentrations or its vehicle (dilute sodium acetate solution) as indicated (n=3 rats per dose with the exception of 10 ng/ml; n=2 rats). Myogenic reactivity is expressed as % change in intraluminal diameter from 60 to 80 mmHg (mean ± SEM). * P≤0.002 vs vehicle by 1-way ANOVA with LSD post-hoc tests.



Supplemental Figure S4. Human relaxin-3, but not Insl-3, inhibits myogenic reactivity of rat small renal arteries. Arteries were isolated and incubated for 3 hr in vitro with (**A**) relaxin-3, (**B**) Insl-3 or rhRLX (all 30 ng/ml) as indicated. In (**A**), some arteries were left untreated or treated with 100μM L-NMMA post-relaxin-3 incubation. Myogenic reactivity is expressed as % increase in intraluminal diameter from 60 to 80 mmHg (mean ± SEM). Relaxin-3, but not Insl-3, significantly inhibited myogenic reactivity in a NOS-dependent manner. Notably, Insl3 pre-treatment did not interfere with rhRLX-induced inhibition of myogenic reactivity (% change in diameter = 8.1 ± 1.8; n=2; data not shown). * $P<0.005$ vs other groups by 1-way ANOVA with LSD post-hoc tests. † $P<0.05$ vs Insl3.