Angiogenic Growth Factors Are New and Essential Players in the Sustained Relaxin Vasodilatory Pathway in Rodents and Humans


Abstract—Relaxin is emerging as an important vasodilator of pregnancy and is being tested for afterload reduction in acute heart failure. However, the mechanisms underlying relaxin-induced vasodilation are incompletely understood. The aims of this study were to establish a new in vitro model for relaxin-induced vasodilation and to use this approach, as well as chronically instrumented, conscious rats, to investigate the role of angiogenic growth factors in the relaxin vasodilatory pathway. Incubation of rat and mouse small renal arteries with recombinant human H2 relaxin for 3 hours in vitro attenuated myogenic constriction, which was blocked by inhibitors of gelatinases, the endothelin B receptor, and NO synthase. These findings corroborate ex vivo observations in arteries isolated from relaxin-infused nonpregnant and midterm pregnant rats, thereby validating the new experimental approach and enabling the study of human arteries. Incubation of small human subcutaneous arteries with relaxin for 3 hours in vitro also attenuated myogenic constriction through the same molecular intermediates. Vascular endothelial growth factor receptor inhibitor SU5416, 3 different vascular endothelial growth factor, and 2 different placental growth factor neutralizing antibodies prevented relaxin from attenuating myogenic constriction in rat and mouse small renal and human subcutaneous arteries. SU5416 administration also prevented relaxin-induced renal vasodilation and hyperfiltration in chronically instrumented, conscious rats. Small renal arteries isolated from these rats demonstrated increased matrix metalloproteinase 2 activity in the relaxin-infused group, which was not prevented by SU5416. We conclude that there is concordance of relaxin vasodilatory mechanisms in rats, mice, and humans, and angiogenic growth factors are novel and essential intermediates. (Hypertension. 2011;57:1151–1160.) ● Online Data Supplement

Key Words: angiogenic growth factors ■ SU5416 ■ small arteries ■ myogenic constriction ■ conscious rats ■ glomerular filtration ■ renal circulation

Relaxin is a 6-kDa protein hormone secreted by the corpus luteum that circulates in the blood during the luteal phase of the menstrual cycle and in pregnancy.1 Evidence is also emerging for a local relaxin ligand/receptor system in arteries.2 Relaxin is a potent vasodilator in many animal species including humans (reviewed in References3 to5). The hormone has also been implicated in the vasodilatory phenomena of pregnancy.6–9 Recent findings suggest that the vasodilatory responses to relaxin are mediated by its major receptor, the relaxin/insulin-like family peptide receptor 1, RXFP1.8 An emerging concept is that the molecular mechanisms of relaxin-induced vasodilation depend on the duration of hormone exposure, that is, rapid (within minutes) and sustained (hours to days). The rapid vasodilatory responses to relaxin are transduced by endothelial G_{q/11} protein coupling to phosphotidylinositol-3 kinase/Akt (protein kinase B)-dependent phosphorylation and activation of NO synthase (NOS), and, unexpectedly, this response is amplified by inhibition of vascular endothelial growth factor (VEGF) receptor tyrosine kinase (RTK) activity.7 Sustained vasodila-
tory responses to relaxin critically depend on increases in arterial gelatinase activity, either matrix metalloproteinase (MMP) 9 or 2, depending on whether the duration of hormone exposure is on the order of hours or days, respectively. The gelatinases, in turn, hydrolyze big endothelin (ET) at a gly-leu bond to form ET-1-32, which activates the endothelial ETB receptor/NO vasodilatory pathway (see Working Model in Figure 7).

Relaxin stimulates VEGF synthesis in several types of fibroblasts, endometrial cells, and macrophages.8–11 Most investigators reported that VEGF increases MMP-2 secretion and activity in cultured human endothelial cells. In addition, VEGF and placental growth factor (PGF) upregulate MMP-9 but not MMP-2 mRNA, protein, and activity in cultured human aortic smooth muscle cells, suggesting an intermediary role for VEGF receptor (R) 1,16,17 Collectively, these findings motivate part of the current work, which is to investigate whether VEGF plays a role in the sustained vasodilatory response to relaxin and, if so, to determine whether it is upstream of the arterial gelatinase(s).

In light of the current trial of recombinant human relaxin (rhRLX) in the treatment of acute heart failure4,18 and its potential therapeutic use in preeclampsia,19,20 both of which capitalize on the hormone’s unique spectrum of vascular effects, revelation of the mechanisms underlying relaxin’s vasodilatory actions is crucial, because it should facilitate these and other future clinical applications.

We first established and extensively validated the use of isolated small arteries in vitro for investigation of the mechanisms of sustained relaxin-induced vasodilation (ie, we incubated small arteries with rhRLX in vitro and then assessed myogenic constriction). This new experimental model is equally robust but considerably more convenient than chronic administration of relaxin to rats.21–24 It also set the stage for translating our investigations in rodents to human arteries. We then tested whether preincubation with the VEGF RTK inhibitor, SU5416, or with specific VEGF and PGF neutralizing antibodies prevents relaxin from attenuating myogenic constriction in rat and mouse small renal and human subcutaneous arteries. To establish in vivo relevance, we next investigated whether the VEGF-R antagonist, SU5416, prevents rhRLX-induced increases in glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) and decreases in effective renal vascular resistance (ERVR) in chronically instrumented, conscious rats. Finally, to determine whether SU5416 prevents the increase in arterial gelatinase activity induced by relaxin, a pivotal step in the relaxin vasodilatory pathway,22 we measured MMP-2 activity in small renal arteries (SRAs) isolated from the chronically instrumented, conscious rats that were studied for renal function.

**Methods**

A full description of procedures may be found in the online Data Supplement. Please see http://hyper.ahajournals.org.

**Myogenic Behavior of Isolated Arteries Treated With rhRLX In Vitro**

The effect of relaxin on myogenic constriction (defined here as the change in arterial diameter at increasing intraluminal pressure from 60 to 80 mm Hg) was assessed in isolated mouse and rat small renal and human subcutaneous arteries as described previously for arteries isolated from gravid or rhRLX-infused rodents.2,21,22,25–27 Human arteries were obtained from subcutaneous fat of patients undergoing elective surgery at the Magee Women’s Hospital, University of Pittsburgh, under the approval of the University of Pittsburgh Institutional Review Board. To determine whether in vitro treatment with rhRLX would attenuate myogenic constriction, arteries were incubated with 30 ng/mL of rhRLX or its vehicle (20 mmol/L of sodium acetate [pH 5.0]) diluted identically as the stock rhRLX) for 3 hours. To test the effect of NOS, ETB receptor, and MMP blockade on rhRLX-mediated reduction of myogenic constriction, the respective inhibitors or their vehicle controls were added to the bath and the arteries incubated for a further 30 minutes before assessment of myogenic constriction. SU5416 and VEGF- and PGF-neutralizing antibodies were added 30 minutes before the 3-hour rhRLX incubation. Note that isotype control antibodies were instilled into the lumen at the same concentration as the neutralizing antibodies, and the latter were documented to be specific (Table S1, available in the online Data Supplement). To account for inherent variability in arterial diameter, as illustrated in Figures S1 and S2 of the online Data Supplement, the results are expressed as the percentage of change in diameter from baseline at 60 mm Hg (ie, no change in diameter indicates relatively robust myogenic constriction, whereas an increase in diameter reflects attenuation of myogenic constriction).

**Renal Function Studies in Conscious Rats**

Rats were chronically instrumented as described previously for assessment of renal function and mean arterial pressure (MAP).28,29 The experimental protocol followed the time line illustrated in Figure 1. Renal function and MAP were assessed in the conscious state as reported previously.28,29

**Zymographic Analysis of SRAs**

After the final renal function experiment, SRAs were isolated and snap frozen for assessment of gelatinase activity by zymography, which was performed as described previously with minor modifications.22,26 The Institutional Animal Care and Use Committee approved all of the procedures involving animals.

**Results**

**Validation of a New Experimental Model for Investigating Mechanisms of Sustained Relaxin-Induced Vasodilation**

SRAs isolated from rats or mice that were administered rhRLX for 5 days showed attenuated myogenic constric-
In most experiments, rat and mouse small renal and human subcutaneous arteries were isolated and incubated with 30 ng/mL of rhRLX for 3 h in vitro. Inhibitor or vehicle was then added to the bath for an additional 30 min (in the continuing presence of rhRLX), after which myogenic constriction was measured. The exception was for NO synthase inhibition with N\(^6\)-monomethyl-L-arginine in mouse small renal arteries, where the arteries were dissected from mice administered \(\mu\)g/h of rhRLX by osmotic pump in vivo for 5 d. Myogenic constriction is expressed as percentage of change in internal diameter from 60 to 80 mm Hg (mean±SEM). See the expanded Methods section for details.

\(^*P<0.05\) vs vehicle. 
\(\dagger P<0.01\) vs vehicle.

**Role of Angiogenic Growth Factors in the Sustained Relaxin Vasodilatory Pathway: Arterial Myogenic Constriction**

Pretreatment of rat small renal and human subcutaneous arteries with the VEGF RTK inhibitor SU5416 (1 \(\mu\)mol/L) for 30 minutes prevented rhRLX (30 ng/mL; 3 hours) from inhibiting myogenic constriction \((P<0.005\) and \(P<0.05\) versus dilute dimethylsulfoxide [DMSO] vehicle control, respectively; Figure 2A and 2B). Thus, myogenic constriction was reduced by rhRLX in the presence of dilute DMSO alone but was preserved when arteries were pretreated with SU5416. Surprisingly, incubation of arteries with SU5416 for 30 minutes after 3-hour treatment with and in the continuing presence of sodium acetate [pH 5.0] diluted identically to the stock rhRLX did not prevent the normal myogenic constriction response of isolated rat small renal and human subcutaneous arteries to a 20-mm Hg increase in intraluminal pressure, either in the presence of inhibitors or their respective vehicles (Table S2). Thus, treatment of small arteries with rhRLX in vitro inhibits myogenic constriction by the same molecular intermediates as described previously for small arteries isolated from rhRLX-treated animals and studied ex vivo.

### Table. Incubation of Isolated Small Arteries With Recombinant Human Relaxin (rhRLX) In Vitro Attenuates Myogenic Constriction Which Is Restored by Subsequent Treatment With Inhibitors of Matrix Metalloproteinases (GM6001), the Endothelin B Receptor (RES-701-1), and NO Synthase (\(N^6\)-Monomethyl-L-Arginine)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rat Small Renal Arteries</th>
<th>Mouse Small Renal Arteries</th>
<th>Human Subcutaneous Arteries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rhRLX + Inhibitor</td>
<td>rhRLX + Vehicle</td>
<td>rhRLX + Inhibitor</td>
</tr>
<tr>
<td>GM6001</td>
<td>0.0±0.3 (n=5 rats)†</td>
<td>9.1±0.9 (n=6 rats)</td>
<td>0.3±0.9 (n=3 mice)*</td>
</tr>
<tr>
<td>RES-701-1</td>
<td>1.6±1.3 (n=6 rats)*</td>
<td>10.2±2.0 (n=6 rats)</td>
<td>2.1±1.1 (n=2 mice)</td>
</tr>
<tr>
<td>(N^6)-Monomethyl-L-arginine</td>
<td>2.3±1.3 (n=6 rats)*</td>
<td>5.8±0.8 (n=6 rats)</td>
<td>1.2±1.0 (n=3 mice)</td>
</tr>
</tbody>
</table>

In most experiments, rat and mouse small renal and human subcutaneous arteries were isolated and incubated with 30 ng/mL of rhRLX for 3 hours in vitro also reduced myogenic constriction (Table). A full-dose response for rhRLX revealed a threshold of \(\approx 6\) ng/mL in rat SRAs (Figure S3). Among other members of the insulin-relaxin family of structurally related hormones, relaxin 3, but not InsL-3, attenuated myogenic constriction of rat SRAs (Figure S4). The addition of GM6001 (1 \(\mu\)mol/L), RES-701-1 (10 \(\mu\)mol/L), or \(N^6\)-monomethyl-L-arginine (100 \(\mu\)mol/L) instead of their respective vehicles to paired arteries from the same animal or patient for 30 minutes after 3-hour rhRLX treatment (and in the continuing presence of rhRLX) abolished the effect of rhRLX and restored myogenic constriction (Table and Figure S4A). Incubation of rat SRAs with a specific MMP-9, but not MMP-2 neutralizing antibody (Ab) also prevented rhRLX from attenuating myogenic constriction (percentage of change in diameter from 60 to 80 mm Hg) in the presence of MMP-9 Ab: 1.6±0.3 [n=6 rats]; MMP-2 Ab: 8.7±0.9 [n=3 rats]; control mouse IgG1k: 8.8±0.9 [n=4 rats]; \(P<0.0001\) MMP-9 versus other treatments by ANOVA with least significant difference post hoc tests). Finally, in essential control experiments, the vehicle for rhRLX (20 mmol/L of sodium acetate [pH 5.0] diluted identically to the stock rhRLX) did not prevent the normal myogenic constriction response of isolated rat small renal and human subcutaneous arteries to a 20-mm Hg increase in intraluminal pressure, either in the presence of inhibitors or their respective vehicles (Table S2). Thus, treatment of small arteries with rhRLX in vitro inhibits myogenic constriction by the same molecular intermediates as described previously for small arteries isolated from rhRLX-treated animals and studied ex vivo.

**Figure 2.** The vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor SU5416 (1 \(\mu\)mol/L) for 30 minutes prevented rhRLX (30 ng/mL; 3 hours) from inhibiting myogenic constriction \((P<0.005\) and \(P<0.05\) versus dilute dimethylsulfoxide [DMSO] vehicle control, respectively; Figure 2A and 2B). Thus, myogenic constriction was reduced by rhRLX in the presence of dilute DMSO alone but was preserved when arteries were pretreated with SU5416. Surprisingly, incubation of arteries with SU5416 for 30 minutes after 3-hour treatment with and in the continuing presence of sodium acetate [pH 5.0] diluted identically to the stock rhRLX did not prevent the normal myogenic constriction response of isolated rat small renal and human subcutaneous arteries to a 20-mm Hg increase in intraluminal pressure, either in the presence of inhibitors or their respective vehicles (Table S2). Thus, treatment of small arteries with rhRLX in vitro inhibits myogenic constriction by the same molecular intermediates as described previously for small arteries isolated from rhRLX-treated animals and studied ex vivo.
rhRLX did not prevent relaxin-induced attenuation of myogenic constriction (P value not significant; Figure 2C).

Consistent with the effect of SU5416, pretreatment of isolated rat and mouse small renal and human subcutaneous arteries with 3 different VEGF-neutralizing antibodies (1 or 3 μg/mL instilled intraluminally) prevented rhRLX from reducing myogenic constriction (P<0.01, <0.001, and <0.05 versus goat IgG, respectively; Figure 3A through 3C). Unexpectedly, a specific PGF-neutralizing Ab (1.0 μg/mL) also blocked the response to rhRLX in isolated mouse and rat SRAs (P<0.01 and P=0.05 versus rat IgG2A, respectively; Figure 3D and 3E). Using another PGF-neutralizing Ab (10 μg/mL), a similar trend for human subcutaneous arteries was clear, although small sample size precluded statistical analysis (Figure 3F). Note that isotype control antibodies were instilled into the lumen at the same concentration as the neutralizing antibodies, and the latter were documented to be specific (Table S1).

We had anticipated that PGF-neutralizing antibodies would serve as additional negative controls along with the isotype control antibodies. To minimize the possibility that the effect of PGF-neutralizing antibodies was because of nonspecific inhibition of VEGF (despite 0% cross-reactivity reported by the manufacturer; Table S1), we also tested a 10-fold lower concentration of PGF Ab in rat SRAs and obtained identical results (percentage of change in diameter with control rat IgG2A/rhRLX: 8.0±1.6 [n=4 rats]; 0.1 μg/mL PGF Ab/rhRLX: −0.4±0.6 [n=5 rats]; P=0.014). Importantly, treatment of isolated rat SRAs with VEGF- or PGF-neutralizing antibodies or SU5416 in this experimental paradigm produced little if any change in the dilatory response to methacholine, suggesting that endothelial function (and, hence, ability of the arteries to dilate in response to rhRLX, which ultimately occurs via endothelial NO) was not compromised (Figure 4). Finally, the vehicle for rhRLX alone had no effect on myogenic constriction of rat SRAs in the presence of either VEGF- or PGF-neutralizing or control antibodies (Table S2).

Role of Angiogenic Growth Factors in the Sustained Relaxin Vasodilatory Pathway: Renal Function in Chronically Instrumented, Conscious Rats

We demonstrated previously that relaxin-induced renal vasodilation and hyperfiltration in chronically instrumented, conscious rats are mediated by arterial gelatinase, the ETB receptor, and NOS2,22–24 that is, the same molecular intermediates that mediate relaxin-induced attenuation of myogenic constriction21,22,26,30 (vide supra). Because our data so far suggested that VEGF and PGF are also involved in mediating reduction of myogenic constriction by relaxin in rat, mouse, and human arteries in vitro, we next tested whether these angiogenic growth factors play a role in relaxin-induced renal
vasodilation and hyperfiltration in vivo. To this end, we administered SU5416 (20 mg/kg per day) or its vehicle (DMSO) SC 24 hours before and then daily during chronic administration of rhRLX (4 µg/h) or its vehicle for 3 to 5 days in conscious, chronically instrumented rats (see Methods section and Figure 1). The circulating concentrations of rhRLX reached in rats administered the hormone were similar between SU5416- and DMSO-treated animals (33.4±4.9 versus 36.9±3.6 ng/mL, respectively; P=0.28 by unpaired t test). These levels are comparable to midterm pregnancy in rats when gestational renal vasodilation and hyperfiltration peak in this species.1,28

In the presence of DMSO (the vehicle for SU5416), rhRLX significantly increased GFR and ERPF and reduced ERVR after 4 to 6 hours and 3 to 5 days of infusion compared with baseline (Figure 5A through 5C, left; all P<0.001 by 1 way ANOVA), as reported previously.23,31 In contrast, SU5416 administration not only prevented the increases in GFR and ERPF and the decrease in ERVR observed after 4 to 6 hours and 3 to 5 days of rhRLX infusion but significantly reduced ERPF and enhanced ERVR (Figure 5A through 5C, left; all P<0.05 versus baseline). rhRLX had no significant effect on MAP in either DMSO- or SU5416-treated rats (Figure 5D, left). Infusion of the vehicle for rhRLX also had no significant effect on GFR, ERPF, ERVR, or MAP at any time point in the presence of either DMSO or SU5416 (Figure 5A through 5D, right).

**Relationship Between Angiogenic Growth Factors and MMP-2 in the Sustained Relaxin Vasodilatory Pathway**

We then investigated gelatinase (MMP-2) activity in SRAs harvested from the rats after the last measurements of renal function and MAP. We reasoned that if angiogenic growth factors are proximal to gelatinases in the sustained relaxin vasodilatory pathway as hypothesized, then SU5416 should block relaxin-induced increases in arterial gelatinase activity. Therefore, MMP-2 activity should be lower in SRAs from SU5416-treated rats. As we reported previously,22,26,30 rhRLX increased both pro- and active MMP-2 activities compared with its vehicle in SRAs from rats that received DMSO, the vehicle for SU5416 (Figure 6A). Expressed as the densitometric ratios of rhRLX:vehicle (whereby a ratio of 1.0 would indicate no difference), the average elevation in total MMP-2 (ie, both pro- and active MMP-2) activities induced by rhRLX was 2.31±0.53 (P=0.028 versus 1.0 by 1 sample t test). However, there was no consistent pattern of change in pro–MMP-2 activity (active MMP-2 band intensity was too low to be quantified for this comparison) between pairs of rhRLX-infused rats (n=9) that were administered DMSO or SU5416 (ratio SU5416:DMSO=1.24±0.26; P=0.39; Figure 6B).

**Discussion**

A major finding of the present work was that treatment of SRAs isolated from rats and mice with rhRLX in vitro attenuates myogenic constriction, thus mimicking the functional behavior of SRAs isolated from rats and mice administered rhRLX by osmotic pump and then studied ex vivo.5,21,22,26,30 Relaxin 3 also attenuates myogenic constriction, most likely through the RXFP1 receptor (although a role for the alternative relaxin 3 receptor RXFP3 was not excluded). In contrast, Insl-3, which signals through the RXFP2 receptor, was inactive alone and also did not prevent rhRLX from attenuating myogenic constriction. This reduction of arterial myogenic constriction by rhRLX corresponds with the renal vasodilatation observed in conscious midterm pregnant and rhRLX-infused nonpregnant rats.22,24,28,29,32

Importantly, we further showed the essential role of arterial gelatinase(s), the endothelial ETB receptor, and NO in rhRLX-induced attenuation of myogenic constriction in vitro. The involvement of these mediators was shown previously for rhRLX- or pregnancy-induced renal vasodilation and for reduction of myogenic constriction of SRAs harvested from rhRLX-infused nonpregnant rats or from gravid rats (reviewed in References5 to8 and20). Also consistent with our previous observation that attenuation of rat SRA myogenic constriction is mediated exclusively by MMP-9 after short-
term (4 to 6 hour) rhRLX infusion, we observed a specific dependency on arterial MMP-9 for reduction of myogenic constriction after 3-hour rhRLX incubation in vitro. In summary, the present studies demonstrate that rhRLX attenuates myogenic constriction of isolated SRAs in vitro in an identical manner to SRAs isolated from gravid rats or nonpregnant rats administered rhRLX and by the same molecular intermediates, thus legitimizing the use of isolated SRAs in vitro to further explore the molecular mechanisms underlying relaxin’s sustained vasodilatory effect. This new experimental model is as robust yet considerably more convenient than earlier approaches because it obviates the need to administer rhRLX in vivo. Therefore, it may accelerate discovery of additional novel relaxin vasodilatory mechanisms. It also provided the opportunity to investigate the effects of relaxin on myogenic constriction in human arteries for the first time.

As such, the discovery that incubation of small subcutaneous arteries from humans with rhRLX for 3 hours in vitro attenuates myogenic constriction, again corresponding with rhRLX-induced renal vasodilation in men and women, was another major finding of this study. Attenuation of myogenic constriction in human subcutaneous arteries in vitro was restored to baseline after blockade of MMPs, the ETB receptor, and NOS, analogous to rodent arteries (see above). Taken together, these findings in rat, mouse, and human small arteries in vitro suggest considerable species conservation of relaxin-induced reduction of myogenic constriction and the underlying molecular mechanisms.

The third major finding is conceptually novel, insofar as we revealed strong evidence for the role of VEGF, and unexpectedly PGF as well, in the sustained relaxin vasodilatory pathway, using the in vitro myogenic constriction bioassay established herein (see above). We discovered that preincubation with the VEGF RTK inhibitor SU5416 blocked attenuation of myogenic constriction by rhRLX in rat and mouse small renal and human subcutaneous arteries. However, SU5416 may not be completely specific for VEGF RTK and could interfere with signal transduction of other growth factor receptors. Rather than using another pharmacological inhibitor that may lack specificity, we took an immunologic approach instead to confirm this finding. Three different VEGF-neutralizing antibodies prevented rhRLX-induced reduction of myogenic constriction in isolated rat and mouse small renal and human subcutaneous arteries, analogous to the results using SU5416.

To demonstrate a unique role for VEGF, we tested 2 different PGF-neutralizing antibodies. Unexpectedly, the
PGF antibodies were equally effective in blocking attenuation of myogenic constriction by rhRLX in isolated small arteries. PGF and VEGF have <45% homology, and all of the antibodies are documented to be specific (Table S1). Nevertheless, we tested a 10-fold lower concentration of the anti-rat/mouse PGF Ab (0.1 μg/mL) to minimize any chance of any cross-reactivity with VEGF. Despite the lower concentration, blockade of rhRLX’s effect on isolated rat SRAs persisted. Thus, vascular PGF serves as an important intermediate in the sustained relaxin vasodilatory pathway, along with VEGF, gelatinase, ETB receptor, and NOS. On the one hand, PGF was reported previously to rapidly vasodilate after starting rhRLX infusion, because we also previously showed nonoverlapping roles for arterial MMP-9 and -2 in sustained relaxin vasodilation and hyperfiltration in chronically instrumented, conscious rats. We reported previously that this is dependent on increased arterial gelatinase activity and activation of the endothelial ETB receptor and NOS.22–24 In the present study, we measured renal function both 4 to 6 hours and 3 to 5 days after starting rhRLX infusion, because we also previously showed that angiogenic growth factors are situated upstream of the endothelial ETB receptor and NOS in the sustained relaxin vasodilatory cascade. That is, treatment of arteries with SU5416 for 30 minutes after 3-hour incubation with rhRLX (and in the continuing presence of rhRLX) is ineffective in restoring myogenic constriction, because distal mechanisms activated during the 3-hour rhRLX incubation period are stable, rendering ongoing VEGF RTK activation unnecessary. In future studies, we will investigate whether myogenic constriction might be restored during longer periods of SU5416 treatment.

Another important facet of this work is that we extended our observation of a critical role for both VEGF and PGF in rhRLX-induced attenuation of myogenic constriction in vitro by testing whether SU5416 prevents rhRLX-induced renal vasodilation and hyperfiltration in chronically instrumented, conscious rats. We reported previously that this is dependent on increased arterial gelatinase activity and activation of the endothelial ETB receptor and NOS.22–24 In the present study, we measured renal function both 4 to 6 hours and 3 to 5 days after starting rhRLX infusion, because we also previously showed nonoverlapping roles for arterial MMP-9 and -2 in the sustained relaxin vasodilatory pathway at these two time points, respectively.22,26,30 Irrespective of the time point, however, relaxin-induced increases in GFR and ERPF, and decreases in ERVR were prevented by VEGF RTK blockade with SU5416. Interestingly, the combination of SU5416 and rhRLX actually resulted in ERPF and ERVR that were significantly below and above baseline, respectively (ie, as measured before administration of either SU5416 or rhRLX). One potential explanation for this observation is that relaxin simultaneously activates both vasodilatory and counterregulatory vasoconstrictor system(s), the latter becoming unmasked when relaxin’s vasodilatory pathway is blocked with SU5416. However, which counterregulatory vasoconstrictor system(s) may be concurrently activated by relaxin and by what mechanisms are presently unknown.

Interestingly, we did not observe either hypertension or albuminuria after 3 to 5 days of SU5416 administration, perhaps because 3 to 5 days is insufficient time to produce hypertension and albuminuria at least with the dose used (20 mg/kg per day SC), and more chronic exposure is required. Because we were interested in whether SU5416 would inhibit “physiological” renal vasodilation elicited by relaxin, we believe it was actually to our advantage to circumvent, albeit serendipitously, these “pathological” effects of SU5416.

Consistent with our previous work, rhRLX administration over several days increased both pro- and active MMP-2 activity in SRAs. Thus, the critical role for arterial gelatinases in sustained relaxin vasodilatory responses is supported by both functional and biochemical evidence22,30 and this study).
However, in contrast to our hypothesis, the increase in MMP-2 induced by rhRLX was not prevented by SU5416. One possible explanation is that the effect of angiogenic growth factors on sustained relaxin-induced vasodilation is indirect and permissive. Another is that VEGF and PGF may be downstream of MMP-2, such that inhibition of these growth factors does not affect the increase in MMP-2 mediated by relaxin. For example, MMP-2 or -9 may release VEGF and/or PGF from the extracellular matrix, which, in turn, are critical to the relaxin vasodilatory pathway by stimulating expression of prepro-ET, as depicted in our working model (Figure 7).

Concordance of results from both in vitro and in vivo approaches strongly supports a role for angiogenic growth factors in the sustained relaxin vasodilatory pathway, but the precise molecular details require further elucidation. Whether relaxin directly affects the expression of VEGF and PGF, their receptors, or soluble receptors and which VEGF receptor mediates relaxin-induced reduction of SRA myogenic constriction are currently under study. Whether PGF binds specifically to VEGF-R1 and not VEGF-R2, and VEGF binds to both receptors, it is tempting to speculate that VEGF-R1 is particularly important. It is also possible that one or both of the angiogenic growth factor coreceptors, neuropilins 1 and 2, are involved. Furthermore, there is considerable potential for cross-talk among the molecular mediators of relaxin vasodilation in arteries. For example, it has been shown that ET can promote VEGF expression in rat aortic smooth muscle cells. The net effect of this positive feedback would be potentiation and propagation of the vasodilatory signal in arteries, which may be important during gestation.

**Perspectives**

A potential limitation of this study is that we did not evaluate myogenic constriction over a range of increasing intraluminal pressures, nor did we evaluate myogenic dilation with decreasing pressure. However, we previously reported the effect of rhRLX administration for 5 days on myogenic constriction of both rat small mesenteric and renal arteries using a broad range of pressures (20 to 120 mm Hg). Myogenic constriction was markedly attenuated by rhRLX virtually throughout the pressure range for both artery types, and in the SRAs, this effect was blocked by NOS inhibition.

Extrapolating from the current work, arterial-derived angiogenic growth factors may be critical to pregnancy-induced renal and systemic vasodilation. This idea is supported by previous studies demonstrating the crucial role of circulating relaxin in maternal vasodilation and increasing global arterial compliance during midterm pregnancy in rats. Considering the marked increases in serum concentrations of placental-derived PGF, particularly during the second half of pregnancy, circulating PGF might synergistically drive maternal vasodilation in late pregnancy, at least partly through the same vasodilatory effectors used by relaxin (ie, the endothelial ETB receptor and NO). In support of this suggestion, preliminary findings in late-pregnant rats indicate that the increase and decrease in global arterial compliance and systemic vascular resistance, respectively, are only partly...
abolished by immunoneutralization of circulating relaxin (K.P.C., unpublished observations, 2008).

Currently, there are no preventative measures, specific therapies, or cures for preeclampsia other than delivery. Thus, there is considerable urgency to evaluate prophylactic, therapeutic, or curative strategies for preeclampsia that emerge from promising preclinical research. To this end, both our current and previous studies of relaxin suggest a therapeutic approach that warrants testing. That is, the vasodilatory properties of administered relaxin should improve arterial function, organ perfusion, and, hence, disease manifestations. From a mechanistic point of view, the hormone might exert salutary effects in preeclampsia by increasing arterial-derigned VEGF and PGF activity, thereby offsetting the deficiency of these angiogenic growth factors in the vascular wall secondary to elevated circulating VEGF-R1.

Acknowledgments

We thank the Department of Pathology Health Sciences Tissue Bank of the University of Pittsburgh Medical Center for assistance with human tissue procurement. We gratefully acknowledge Wei Hou PhD, Assistant Professor, Department of Health Outcomes Policy, University of Florida College of Medicine, for assistance with statistical analysis. The human relaxin 3 and Insl-3 peptides were synthesized by Akhter Hossain and Suode Zhang and kindly provided by John D. Wade (Howard Florey Institute, University of Melbourne, Melbourne, Victoria, Australia).

Sources of Funding

This work was supported by National Institutes of Health grants ROI DK063321, ROI HL067937, and R21 HL093605; American Heart Association Grant-in-Aid 0855090E, and an American Heart Association postdoctoral fellowship (to J.T.M.).

Disclosures

K.P.C. holds patents for relaxin.

References

Angiogenic Growth Factors Are New and Essential Players in the Sustained Relaxin Vasodilatory Pathway in Rodents and Humans

Hypertension. 2011;57:1151-1160; originally published online May 2, 2011;
doi: 10.1161/HYPERTENSIONAHA.110.165027
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/57/6/1151

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2011/04/29/HYPERTENSIONAHA.110.165027.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

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

Jonathan T. McGuane¹, Leslie A. Danielson³, Julianna E. Debrah⁴, J. Peter Rubin⁵, Jacqueline Novak⁶, and Kirk P. Conrad¹,²

¹Departments of Physiology and Functional Genomics, and ²Obstetrics and Gynecology, University of Florida College of Medicine, and D.H. Barron Reproductive and Perinatal Biology Research Program, Gainesville FL; ³Department of Pathology, University of New Mexico School of Medicine, Albuquerque NM; ⁴Magee-Womens Research Institute, and ⁵Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh PA; ⁶Division of Math and Sciences, Walsh University, North Canton OH.

Corresponding author: Jonathan T. McGuane
Department of Physiology and Functional Genomics,
and D.H. Barron Reproductive and Perinatal Biology Research Program
University of Florida College of Medicine
1600 SW Archer Road
M552, PO Box 100274
Gainesville, FL 32610-0274
Phone 352-392-3709 Fax 352-846-0270
Email jtmcguane@ufl.edu

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 Ca²⁺-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 N⁶-monomethyl-L-arginine acetate (L-NMMA, a competitive inhibitor of NOS; Sigma-Aldrich) (8), 10 μmol/L RES-701-1 (ETB 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 Kruscal-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 ETB receptor antagonist RES-701-1 or the mixed ETA/B antagonist SB209670, but not the ETA 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 ETB 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 ((D80-D60)/D60×100 %). 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 Ca2+-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} × 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.
References


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

<table>
<thead>
<tr>
<th>Neutralizing antibody</th>
<th>Control antibody</th>
<th>Concentration</th>
<th>Cross Reactivity #</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-mouse VEGF (R&amp;D Systems Cat# AF-493-NA)</td>
<td>goat IgG</td>
<td>1 μg/ml *</td>
<td>0 - &lt;5% rhPGF; 0% rmPGF-2</td>
</tr>
<tr>
<td>anti-human VEGF (R&amp;D Systems Cat# AF-293-NA)</td>
<td>goat IgG</td>
<td>1 μg/ml *</td>
<td>0% rhPGF</td>
</tr>
<tr>
<td>anti-rat VEGF (R&amp;D Systems Cat# AF-584)</td>
<td>goat IgG</td>
<td>3 μg/ml *</td>
<td>0% rhPGF &amp; rmPGF-2</td>
</tr>
<tr>
<td>anti-mouse and rat PGF-2 (R&amp;D Systems Cat# MAB465) †</td>
<td>rat IgG &lt;sub&gt;2A&lt;/sub&gt;</td>
<td>1.0 and 0.1 μg/ml *</td>
<td>0% rhVEGF and rhPGF</td>
</tr>
<tr>
<td>anti-human PGF (R&amp;D Systems Cat# MAB284)</td>
<td>mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10 μg/ml ‡</td>
<td>0% rhVEGF</td>
</tr>
<tr>
<td>Anti-human rat and mouse MMP-2 (Chemicon Cat# MAB13405)</td>
<td>mouse IgG&lt;sub&gt;k&lt;/sub&gt;</td>
<td>3 μg/ml §¶</td>
<td></td>
</tr>
<tr>
<td>Anti-rat and mouse MMP-9 (Lab Vision Cat# MS-819-p1ABX)</td>
<td>mouse IgG&lt;sub&gt;k&lt;/sub&gt;</td>
<td>10 μg/ml ¶¶</td>
<td></td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th></th>
<th>Vehicle for rhRLX + vehicle or control Ab</th>
<th>Vehicle for rhRLX + inhibitor or neutralizing Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM6001</td>
<td>1.6 ± 0.6 (n=3 rats)</td>
<td>1.4 ± 0.6 (n=3 rats)</td>
</tr>
<tr>
<td>RES-701-1</td>
<td>0.0 ± 1.0 (n=3 rats)</td>
<td>1.1 ± 1.5 (n=3 rats)</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>2.3 ± 0.1 (n=2 rats)</td>
<td>0.6 ± 1.4 (n=3 rats)</td>
</tr>
<tr>
<td>VEGF Ab</td>
<td>1.5 ± 2.1 (n=4 rats)</td>
<td>-0.4 ± 0.7 (n=4 rats)</td>
</tr>
<tr>
<td>PIGF Ab</td>
<td>1.1 ± 1.3 (n=3 rats)</td>
<td>1.3 ± 1.0 (n=3 rats)</td>
</tr>
</tbody>
</table>

Human subcutaneous arteries

<table>
<thead>
<tr>
<th></th>
<th>Vehicle for rhRLX + vehicle</th>
<th>Vehicle for rhRLX + inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NMMA</td>
<td>-0.8 ± 0.6 (n=5 patients)</td>
<td>-0.7 ± 0.6 (n=5 patients)</td>
</tr>
</tbody>
</table>

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 Ca²⁺-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 ± 0.6 µm) and C (Δ 11.6 ± 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 Ca²⁺-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.