Relaxin regulates vascular wall remodeling and passive mechanical properties in mice

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Submitted 27 July 2010; accepted in final form 28 April 2011

Debrab DO, Debrab JE, Haney JL, McGuane JT, Sacks MS, Conrad KP, Shroff SG. Relaxin regulates vascular wall remodeling and passive mechanical properties in mice. J Appl Physiol 111: 260–271, 2011. First published May 5, 2011; doi:10.1152/japplphysiol.00845.2010.—Administration of recombinant human relaxin (rhRLX) to conscious rats increases global arterial compliance, and small renal arteries (SRA) isolated from these rats demonstrate increased passive compliance. Here we characterize relaxin-induced vascular remodeling and examine its functional relevance. SRA and external iliac arteries (EIA) were examined in rhRLX-treated (1.0 μg/h for 5 days) and relaxin knockout mice. Arterial geometric remodeling and compositional remodeling were quantified using immunohistochemical and biochemical techniques. Vascular mechanical properties were quantified using an in vivo preparation wherein pressure-diameter data were obtained at various axial lengths. Compared with vehicle-treated mice, SRA from rhRLX-treated mice showed outward geometric remodeling (increased unstressed wall area and wall-to-lumen area ratio), increased smooth muscle cell (SMC) density, reduction in collagen-to-total protein ratio, and unchanged elastin-to-tissue dry weight ratio. Compared with wild-type mice, relaxin knockout mice exhibited the opposite pattern: decreased unstressed wall area and wall-to-lumen area ratio, decreased SMC density, and increased collagen-to-total protein ratio. Although tissue biaxial strain energy of SRA was not different between rhRLX- and vehicle-treated groups at low-to-physiological circumferential and axial strains, it was lower for the rhRLX-treated group at the highest circumferential strain. In contrast to SRA, relaxin administration was not associated with any vascular remodeling or changes in passive mechanics of EIA. Thus relaxin induces both geometric and compositional remodeling in vessel-specific manner. Relaxin-induced geometric remodeling of SRA is responsible for the increase in passive compliance under low-to-physiological levels of circumferential and axial strains, and compositional remodeling becomes functionally relevant only under high circumferential strain.

arterial compliance; arterial collagen; elastin; smooth muscle; matrix metalloproteinase-2; relaxin knockout mice

Relaxin is a 6-kDa peptide hormone that emanates from the corpus luteum and circulates during the luteal phase of the menstrual cycle and in pregnancy (42). Traditionally, the hormone has been associated with the female reproductive tract, but recent evidence indicates that relaxin plays an important role in cardiovascular function (reviewed in Refs. 7, 28). We found that administration of recombinant human relaxin (rhRLX) to chronically instrumented, nonpregnant female and male rats increased cardiac output and global arterial compliance (AC), as well as decreased systemic vascular resistance (6, 13, 14). Concurrent with relaxin’s influence on overall cardiovascular function, small renal arteries isolated from the rhRLX-treated, nonpregnant female rats demonstrated increased passive compliance (6). More recently, we explored the role of endogenous, vascular-derived relaxin in modifying arterial mechanical properties in mice. We observed that small renal arteries isolated from relaxin knockout (RLX−/−) mice are characterized by decreased passive compliance compared with arteries from wild-type (RLX+/+) control mice (33).

The mechanistic bases for the relaxin-induced increase in global AC are not fully understood. However, it is likely that the increase in passive compliance of arteries resulting from relaxin-mediated vascular wall remodeling contributes to the overall increase in global AC. First, small renal arteries from relaxin-treated rats and RLX−/− mice have increased and decreased vascular wall area, respectively, compared with the appropriate control group (6, 33). Second, relaxin increases vascular gelatinase activity in small renal and other arteries when administered to nonpregnant rats (25–27, 50) and vascular gelatinase has been implicated in vascular wall remodeling through degradation of extracellular matrix proteins such as collagen and elastin (29). Based on these findings, we hypothesized that the relaxin-induced increase in global AC is at least partly due to the hormone’s ability to alter passive AC through its actions (either directly or indirectly) on the smooth muscle cells (SMCs) and matrix in the vascular wall. Thus the first aim of this study was to determine the effects of relaxin on 1) geometric remodeling of the vascular wall in relation to SMC density, and 2) compositional remodeling focusing on collagen and elastin contents in small renal arteries. The second aim was to determine the relative contributions of geometric and compositional remodeling to the increase in passive AC observed following relaxin administration. Of note, in our preliminary work examining the effect of relaxin on passive AC and vascular wall area, we found that external iliac arteries did not exhibit relaxin-induced changes in passive compliance and wall area (unpublished observations). These findings suggest that the vascular effects of relaxin may be artery-type specific. Thus the third aim of the current study was to determine the...
vascular wall remodeling effects of relaxin, if any, on the external iliac artery.

MATERIALS AND METHODS

Ethical Approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Magee Women's Research Institute, University of Pittsburgh.

Animals

Female C57Bl/6J mice of 8–10 wk of age (body weight: ~20 g) were purchased from Harlan Laboratories (Frederick, MD). Male wild-type [RLX\(^{+/+}\); body weight (means ± SE): 25.1 ± 0.4 g; n = 14] and relaxin knockout (RLX\(^{-/-}\); body weight: 24.8 ± 0.5 g; n = 12) mice 8- to 12-wk-old were generously provided by Dr. Laura J. Parry, (University of Melbourne, Melbourne, Australia). Animals were provided PROLAB RMH 2000 diet containing 0.48% sodium (PME Feeds, St. Louis, MO) and water ad libitum.

Administration of rhRLX

rhRLX was administered at 1.0 μg/h over 5 days by model 1002 osmotic minipumps (Durect, Cupertino, CA) that were inserted subcutaneously in the back of the animal under isoflurane anesthesia. The hormone (courtesy of Elaine Unemori, Corthera, San Mateo, CA) was provided 60 mg/kg pentobarbital intraperitoneally. For isolation of external iliac arteries, mice were anesthetized with 60 mg/kg pentobarbital intraperitoneally. For isolation of small renal arteries, a kidney was removed and placed in HEPES-buffered physiological salt solution (PSS) at room temperature. A stereo dissecting microscope, fine forceps, and iridectomy scissors were used to isolate interlobar arteries as described by Gandel et al. (20). Briefly, the arteries were exposed by careful removal of the overlying medullary tissue. The main renal artery typically divides into three to four branches in each half of the kidney that course to the renal cortex. These branches were gently dissected from the surrounding medullary tissue, and the distal segments were used for the functional studies. For isolation of external iliac arteries, mice were placed in the supine position and incisions were made in both hindlimbs to expose the iliac arteries. Segments of the external iliac arteries with the surrounding tissue were then removed and placed in HEPES-buffered PSS. The arterial segments were then carefully dissected from extraneous tissue.

Passive Mechanical Properties of Vascular Wall: Biaxial Characterization

After isolation, the arterial segment was transferred to an isotonic arteriograph (Living Systems Instrumentation, Burlington, VT), with the chamber bath containing 0.0001 M papaverine and 0.01 M EGTA in calcium-free HEPES-buffered PSS at 37°C (34). The specimen holder was modified such that luminal pressure–diameter relationship and axial force could be recorded at various axial lengths. Measured luminal pressure–diameter and axial force data were used to characterize biaxial mechanical properties of vessel wall tissue as described by Schulze-Bauer et al. (41). Details regarding the experimental setup, protocol, and data acquisition and analysis are presented in APPENDIX A.

Biochemical and Histological Measurements

The details regarding various biochemical and histological assays and measurements are presented in APPENDIX B. Briefly, biochemical assays were used to quantify vascular wall total protein, collagen, and elastin contents, as well as vascular gelatinase activity (gelatin zymography). To quantify vascular SMC density, α-smooth muscle actin and nuclei were colocalized in fixed tissue sections using immunofluorescence and Hoescht dye, respectively. Figure 1 shows a representative fluorescence image of a fixed tissue section that was analyzed to quantify vascular SMC density (no. of cells/1,000 μm\(^2\) wall area).

Statistical Analysis

Data are expressed as means ± SE. Student’s unpaired t-test was used to compare mean values between the two groups: rhRLX-treated and vehicle-treated (control), or RLX\(^{-/-}\) and RLX\(^{+/+}\) animals. Two-way ANOVA with repeated measures on one factor was used to compare strain energies between the two groups (factor 1, control vs. rhRLX treated) at various combinations axial and circumferential strains (factor 2, 9 combinations, repeated measures). Post hoc pairwise comparisons were performed using Fisher’s least significant difference method. A P value <0.05 was considered significant.

RESULTS

Geometric Remodeling

Exogenous relaxin administration. With the use of pressure arteriograph, small renal arteries isolated from rhRLX-treated mice demonstrated no difference in lumen area (Fig. 2B), but they showed a trend towards increased unstressed wall area (Fig. 2A; P = 0.08) and increased wall-to-lumen area ratio (Fig. 2C; P = 0.06) compared with the vehicle-treated group. Immunohistochemical data indicated the same pattern of geometric remodeling (rhRLX treated, n = 7 mice, vs. vehicle treated, n = 6 mice): unstressed lumen area (5,173 ± 320 vs. 4,847 ± 129 μm\(^2\); P = NS); unstressed wall area (5,688 ± 258 vs. 4,383 ± 225 μm\(^2\); P = 0.005); and unstressed wall-to-lumen area ratio (1.12 ± 0.07 vs. 0.91 ± 0.05; P = 0.03). It should be noted that absolute values of unstressed vascular...
lumen and wall areas by histology were significantly smaller than those by pressure arteriograph (Fig. 2, A and B). We believe that this is primarily due to the fact that smaller renal arteries were (unintentionally) chosen for histological analyses and secondarily to fixation-induced tissue shrinkage. However, the patterns of rhRLX-induced changes in areas and absolute values of unstressed lumen-to-wall area ratios were not different between the two methods. Finally, SMC density was significantly higher in the rhRLX-treated group (Fig. 2A).

These data indicate that exogenous relaxin administration resulted in SMC hyperplasia, which contributed to the increases in small renal artery unstressed wall area and wall-to-lumen area ratio.

In contrast to the small renal artery, rhRLX administration did not cause any geometrical remodeling of iliac arteries as measured by pressure arteriograph (rhRLX treated, n = 10 mice, vs. vehicle treated, n = 11 mice): unstressed lumen area (24,429 ± 1,581 vs. 23,680 ± 1,278 μm²; P = NS); unstressed arterial wall area (21,683 ± 1,664 vs. 20,828 ± 1,345 μm²; P = NS); and unstressed wall-to-lumen area ratio (0.95 ± 0.11 vs. 0.97 ± 0.10; P = NS). This lack of geometric remodeling was further supported by the immunohistochemical data (rhRLX treated, n = 6 mice, vs. vehicle treated, n = 5 mice): unstressed lumen area (7,907 ± 276 vs. 7,908 ± 294 μm²; P = NS); unstressed wall area (7,272 ± 319 vs. 7,330 ± 486 μm²; P = NS); and unstressed wall-to-lumen area ratio (0.92 ± 0.04 vs. 0.93 ± 0.05; P = NS). SMC density of iliac arteries was also unaffected by rhRLX administration (rhRLX treated, n = 6 mice, vs. vehicle treated, n = 5 mice; 3.9 ± 0.2 vs. 4.0 ± 0.2 cells/1,000 μm²; P = NS).

Endogenous relaxin. The use of pressure arteriograph, small renal arteries isolated from RLX−/− mice were demonstrated unchanged lumen area (Fig. 3B) and significantly decreased unstressed wall area (Fig. 3A) and wall area-to-lumen area ratio (Fig. 3C). Immunohistochemical data indicated the same pattern of geometric remodeling (RLX−/−, n = 6 mice, vs. RLX+/+, n = 4 mice): unstressed lumen area (4,689 ± 262 vs. 4,841 ± 405 μm²; P = NS); unstressed wall area (3,681 ± 136 vs. 4,467 ± 244 μm²; P = 0.03); and unstressed wall-to-lumen area ratio (0.79 ± 0.04 vs. 0.93 ± 0.03; P = 0.03). SMC density of small renal arteries was significantly lower in the RLX−/− group (Fig. 3D). Thus renal arterial geometric responses to loss of endogenous relaxin are opposite to those observed after exogenous relaxin administration.

For the external iliac artery, immunohistochemical data indicated that there were no differences between the two groups (RLX−/−, n = 4 mice vs. RLX+/++; n = 5 mice): unstressed lumen area (7,875 ± 441 vs. 7,557 ± 432 μm²; P = NS); unstressed wall area (7,187 ± 172 vs. 6,990 ± 273 μm²; P = NS); unstressed wall-to-lumen area ratio (0.92 ± 0.03 vs. 0.94 ± 0.06; P = NS); and SMC density (3.6 ± 0.4 vs. 3.8 ± 0.7 cells/1,000 μm²; P = NS). Because isolated vascular mechanics experiments were not performed with external iliac arteries from RLX−/− and RLX+/+ mice, pressure arteriograph-based geometric data are not available.

Compositional Remodeling

Exogenous relaxin administration. Small renal arteries from rhRLX-treated mice were characterized by a significant reduction in the collagen-to-total protein ratio compared with vehicle-infused, control mice (Fig. 2E), with no significant difference in elastin-to-tissue dry weight ratio (Fig. 2F). In contrast, external iliac arteries from rhRLX-treated mice did not exhibit any significant differences in collagen-to-total protein ratio (rhRLX treated: 0.25 ± 0.02 μg collagen/μg protein, n = 5 mice; vehicle treated: 0.28 ± 0.04 μg collagen/μg protein, n = 5 mice; P = NS) or elastin-to-tissue dry weight ratio compared with vehicle-infused, control mice (rhRLX treated: 89 ± 12 μg elastin/mg dry weight, n = 4 mice; vehicle treated: 83 ± 5 μg elastin/mg dry weight, n = 5 mice; P = NS).

Endogenous relaxin. Small renal arteries isolated from RLX−/− mice exhibited significantly increased collagen-to-total protein ratio compared with RLX+/+ mice (Fig. 3D).
Although there was a trend towards increased collagen-to-total protein ratio for external iliac arteries from RLX\textsuperscript{+/+} mice compared with RLX\textsuperscript{−/−} mice, the difference did not reach statistical significance (RLX\textsuperscript{+/+}: 0.41 ± 0.05 μg collagen/μg protein, \(n = 5\) mice; RLX\textsuperscript{−/−}: 0.30 ± 0.04 μg collagen/μg protein, \(n = 6\) mice; \(P = 0.06\)). Due to the insufficient quantity of samples, we were unable to assess elastin content of small renal and external iliac arteries of RLX\textsuperscript{+/+} and RLX\textsuperscript{−/−} mice.

Passive Mechanical Properties of Vascular Wall: Biaxial Characterization

Arterial passive mechanical properties. To this point, our findings indicate that relaxin induces two types of vascular wall remodeling in small renal arteries: geometric and compositional. We have previously reported that small renal arteries from rhRLX-treated rats are also characterized by an increase in passive compliance and unstressed wall area. To determine the relative contributions of these two forms of relaxin-induced vascular remodeling to the increase in passive compliance, we examined the passive vascular wall mechanical properties of small renal arteries from rhRLX- and vehicle-treated, control mice. Isolated arteries were subjected to pressure cycles from 0 to 120 mmHg at various axial stretches, and vascular wall mechanical properties were quantified in terms of tissue strain energy (see MATERIALS AND METHODS). A representative mechanical response of an artery under multidimensional loading conditions is illustrated in Fig. 4.

We tested several different strain-energy functions and found that the four-parameter polynomial model adapted from Vaishnav et al. (49) best described our data (Tables 1 and 2), i.e., excellent fits for stress-strain data (Fig. 4) resulting in high coefficient of determination values (\(R^2 > 0.93\)) and good model parameter estimation (i.e., none of the parameter 95% confidence intervals included zero).

Vascular wall mechanical behavior of each small renal artery sample was quantified in terms of the calculated strain energy surface. Specifically, estimated model parameters for each sample (Tables 1 and 2) and Eq. A9 were used to calculate tissue strain energy for a range of axial (\(E_{zz}\)) and circumferential (\(E_{\theta\theta}\)) Green-Lagrange strains. We used common strain ranges that were representative of our experimental conditions (\(E_{zz}: 0.40\) to 0.90 and \(E_{\theta\theta}: 0.05\) to 1.50). Individual strain energy surfaces within each group were averaged, and the difference in strain energy surfaces between the two groups
RELAXIN, VASCULAR WALL REMODELING, AND PASSIVE MECHANICS

Table 1. Best fit constitutive parameters for small renal arteries isolated from vehicle-treated mice

<table>
<thead>
<tr>
<th>Vessel</th>
<th>(C_1)</th>
<th>(C_2)</th>
<th>(C_3)</th>
<th>(C_4)</th>
<th>(R^2(\sigma_{00}))</th>
<th>(R^2(\sigma_{zz}))</th>
</tr>
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<tr>
<td>SRA #1</td>
<td>11,130</td>
<td>12,751</td>
<td>7,780</td>
<td>14,398</td>
<td>0.94</td>
<td>0.94</td>
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<td></td>
<td>(9,267, 12,992)</td>
<td>(10,181, 15,321)</td>
<td>(6,479, 9,081)</td>
<td>(11,224, 17,573)</td>
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<td>11,821</td>
<td>13,408</td>
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<td>11,618</td>
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<tr>
<td></td>
<td>(10,480, 13,163)</td>
<td>(12,000, 14,976)</td>
<td>(8,058, 9,605)</td>
<td>(9,557, 13,680)</td>
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<tr>
<td>SRA #3</td>
<td>7,313</td>
<td>7,787</td>
<td>4,275</td>
<td>11,589</td>
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<td>0.95</td>
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<td></td>
<td>(6,025, 8,600)</td>
<td>(6,116, 9,458)</td>
<td>(3,459, 5,092)</td>
<td>(9,830, 13,349)</td>
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<tr>
<td>SRA #4</td>
<td>8,145</td>
<td>8,985</td>
<td>3,968</td>
<td>9,798</td>
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<td></td>
<td>(6,975, 9,315)</td>
<td>(7,685, 10,285)</td>
<td>(3,336, 4,636)</td>
<td>(8,255, 11,341)</td>
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<td>11,071</td>
<td>7,116</td>
<td>11,466</td>
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<td>(7,758, 10,256)</td>
<td>(8,640, 13,502)</td>
<td>(5,932, 8,300)</td>
<td>(9,019, 13,914)</td>
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<tr>
<td>SRA #6</td>
<td>10,819</td>
<td>14,064</td>
<td>7,563</td>
<td>12,831</td>
<td>0.95</td>
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<tr>
<td></td>
<td>(9,465, 12,173)</td>
<td>(11,419, 16,710)</td>
<td>(6,254, 8,872)</td>
<td>(10,199, 15,463)</td>
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<td>SRA #7</td>
<td>10,533</td>
<td>10,140</td>
<td>4,963</td>
<td>12,197</td>
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<td>0.97</td>
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<td>(8,411, 12,655)</td>
<td>(8,162, 12,118)</td>
<td>(4,041, 5,885)</td>
<td>(9,516, 14,878)</td>
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<tr>
<td>Mean</td>
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<td>11,184</td>
<td>6,359</td>
<td>11,985</td>
<td>0.95</td>
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<td>894</td>
<td>725</td>
<td>533</td>
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\(C_1-C_4\), best-fit constitutive parameters for each artery computed by fitting original pressure-diameter and force-length data to a 4-parameter polynomial model of biaxial strain energy using the Levenberg-Marquardt algorithm; values in parentheses represent 95% confidence intervals for each estimated parameter. \(R^2(\sigma_{00})\) and \(R^2(\sigma_{zz})\), coefficients of determination calculated for the fits to circumferential and axial second Piola-Kirchoff stresses, respectively. SRA, small renal artery.

Table 2. Best fit constitutive parameters for small renal arteries isolated from recombinant human relaxin-treated mice

<table>
<thead>
<tr>
<th>Vessel</th>
<th>(C_1)</th>
<th>(C_2)</th>
<th>(C_3)</th>
<th>(C_4)</th>
<th>(R^2(\sigma_{00}))</th>
<th>(R^2(\sigma_{zz}))</th>
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<td>SRA #8</td>
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<td>10,026</td>
<td>7,158</td>
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<td>(7,025, 9,139)</td>
<td>(8,401, 11,651)</td>
<td>(6,337, 7,980)</td>
<td>(10,722, 14,223)</td>
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<tr>
<td>SRA #9</td>
<td>6,788</td>
<td>6,729</td>
<td>4,080</td>
<td>8,607</td>
<td>0.96</td>
<td>0.93</td>
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<tr>
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<td>(5,780, 7,796)</td>
<td>(6,094, 8,384)</td>
<td>(3,490, 4,670)</td>
<td>(7,109, 10,106)</td>
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<td></td>
</tr>
<tr>
<td>SRA #10</td>
<td>8,232</td>
<td>11,398</td>
<td>7,070</td>
<td>12,035</td>
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<td>0.97</td>
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<td>(7,268, 9,196)</td>
<td>(9,171, 13,626)</td>
<td>(6,559, 8,855)</td>
<td>(10,142, 13,928)</td>
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<tr>
<td>SRA #11</td>
<td>7,323</td>
<td>12,752</td>
<td>12,657</td>
<td>7,947</td>
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<td>(6,539, 8,106)</td>
<td>(10,525, 14,979)</td>
<td>(11,451, 13,863)</td>
<td>(6,326, 9,569)</td>
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<tr>
<td>SRA #12</td>
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<td>8,352</td>
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<td>(6,275, 9,253)</td>
<td>(6,944, 9,161)</td>
<td>(2,387, 3,452)</td>
<td>(9,270, 12,592)</td>
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<td>5,801</td>
<td>14,302</td>
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<td>(9,951, 12,693)</td>
<td>(10,727, 13,514)</td>
<td>(5,116, 6,487)</td>
<td>(12,220, 16,385)</td>
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<td>SRA #14</td>
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<td>10,489</td>
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<td>(7,281, 9,817)</td>
<td>(6,613, 9,759)</td>
<td>(7,299, 8,827)</td>
<td>(8,509, 12,470)</td>
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<tr>
<td>Means</td>
<td>8,294</td>
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<td>6,912</td>
<td>10,969</td>
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<td>SE</td>
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Abbreviations and other explanations are same as in Table 1.

Vascular Gelatinase Activity

Exogenous relaxin administration. Representative zymograms for the three different artery types that were isolated from mice administered rhRLX or vehicle for 5 days are depicted in Fig. 8A. The data are summarized in Fig. 8B. There were significant, albeit small, decreases in pro-matrix metalloproteinase (MMP)-2 and pro-MMP-9 in the mesenteric arteries from the rhRLX-treated compared with vehicle-treated mice. In contrast, there were no significant differences in gelatinase activities in the small renal arteries or aortas between the two groups of animals.

DISCUSSION

The major objectives of the present work were to determine 1) the effects of relaxin on cellular and biochemical constituents of the vascular wall, and 2) the relative contributions of (vehicle treated and rhRLX treated) is illustrated in Fig. 5. We found that there was little or no difference in strain energy under conditions of low \(E_{zz}\), regardless of the level of \(E_{zz}\) (Figs. 5 and 6A). However, as \(E_{zz}\) increased, there was a trend of increasingly lower levels of strain energy in the small renal arteries from the rhRLX-treated group compared with the vehicle-treated group (Fig. 5), with the difference reaching statistical significance at the highest level of \(E_{zz}\) (Fig. 6A). For the chosen strain energy function (Eq. A9), the axial tangent modulus (\(TM_{zz}\)) is strain independent (Eq. A9) and circumferential (\(TM_{00}\)) and cross (\(TM_{zz}\) or \(TM_{00}\)) tangent moduli are dependent on \(E_{zz}\) (Eq. A12) and \(E_{00}\) (Eq. A14), respectively. \(TM_{zz}\) was not different between the two groups (Fig. 6B). \(TM_{00}\) and \(TM_{zz}\) (or \(TM_{00}\)) were consistently lower for the rhRLX-treated group at all strains, reaching statistical significance at the highest strain levels (Fig. 6, C and D).

Next, we examined the biaxial mechanical behavior of external iliac arteries from rhRLX-treated and vehicle-treated mice using similar techniques as above. Consistent with the lack of effect of relaxin on the collagen content, we found that there was no relaxin-induced change in tissue strain energy of external iliac arteries under any loading conditions (Fig. 7).
the different forms of relaxin-induced vascular wall remodeling to the increase in passive AC observed following relaxin administration. We report here that relaxin induces both outward geometric (increased unstressed wall area and wall area-to-lumen area ratio) and compositional (decreased collagen-to-total protein ratio) remodeling in small renal arteries but not external iliac arteries. Furthermore, the relaxin-induced compositional remodeling of small renal arteries becomes functionally relevant only under conditions of high circumferential strains.

**Relaxin-Induced Compositional Remodeling of the Vascular Wall**

The increase in vascular gelatinases observed after administration of rhRLX to nonpregnant rats led us to consider that relaxin may induce structural remodeling of the vascular wall since vascular gelatinase activity has been implicated in vascular remodeling through degradation of extracellular matrix proteins such as collagen and elastin (29). We postulated that, along with the reduction in smooth muscle tone (and possibly angio- or neovascularogenesis), arterial remodeling also contributes to the increase in global AC in vivo observed after relaxin administration to nonpregnant rats (6). We found relaxin induces compositional remodeling of small renal arteries isolated from mice by reducing collagen-to-total protein ratio. This finding is consistent with several other published reports implicating relaxin’s ability to antagonize the synthesis of or promote degradation of collagen. At the cellular level, rhRLX treatment has been shown to reduce the expression of collagen in human dermal fibroblasts and neonatal rat fibroblasts following stimulation with TGF-β1 (32, 47). At the tissue level, rhRLX treatment has been shown to reduce collagen content in rodent models of renal, pulmonary, hepatic, and cardiac fibrosis (21, 32, 37, 48, 51). Additionally, administration of rhRLX to hypoxic rats reduced accumulation of collagen in the main pulmonary artery (44). Relaxin-deficient (RLX−/−) mice have also been shown to exhibit an accelerated, age-related increase in collagen content in the heart, lung, kidneys, and skin (17, 36, 38–40). These findings in RLX−/− mice are consistent with our current work in which we found that small renal arteries from RLX−/− mice exhibit significantly increased levels of collagen-to-total protein ratio compared with control counterparts. Furthermore, administration of rhRLX to older RLX−/− mice significantly reversed the established fibrosis in lungs, kidneys, and heart (36). Xu et al. (52) recently reported comparable results in relaxin-treated senescent spontaneously hypertensive rats, i.e., the hormone increased SMC density and reduced collagen in the aorta.

Unexpectedly, relaxin infusion did not enhance MMP-2 or MMP-9 (gelatinase) activity in small renal arteries, mesenteric resistance arteries, and aorta from mice, which is in contrast to our previous studies using rats (25–27, 31). Instead, relaxin actually decreased both pro-MMP-2 and pro-MMP-9 activities, albeit slightly, in mesenteric vessels (Fig. 8B). This discrepancy with the results previously obtained in rat arteries may arise from a different mode of action for relaxin in murine vasculature. Because the inhibitory effect of relaxin on myogenic constrictor in mouse small renal arteries was blocked by the general MMP inhibitor GM6001 (31), one possible explanation for these discrepant findings is that one or more MMPs besides the gelatinases are crucial to relaxin-induced inhibition of myogenic constriction and arterial compositional remodeling in mouse small renal arteries. Another possibility is that gelatinase(s) activity in situ may actually be increased due to reduced levels of tissue inhibitors of matrix metalloproteinases, which we did not evaluate in this study. Finally, relaxin has also been shown to inhibit stimulated collagen gene expression (47), and this might be the main mechanism for the decrease in collagen content observed in relaxin-treated mice or relaxin wild-type compared with knockout mice.

**Relaxin-Induced Geometric Remodeling of the Vascular Wall**

Small renal arteries isolated from rhRLX-treated mice demonstrated outward geometric remodeling (i.e., increased unstressed wall area and wall-to-lumen area ratio) compared with arteries from vehicle-infused animals. Similarly, small renal arteries harvested from RLX−/− mice showed significantly increased unstressed wall area and wall area-to-lumen area ratio compared with RLX−/− mice. Of note, geometric remodeling of the vascular wall has also been reported in pregnant animals. Osol and Cipolla (35) reported that uterine radial arteries from late pregnant rats demonstrated a significant increase in vascular wall cross-sectional area compared with those from nonpregnant rats. In subsequent work, they showed that vascular SMCs isolated from radial arteries were significantly elongated during late gestation and that the rate of proliferation of these cells and those from the main uterine artery was significantly increased. Accordingly, they concluded that both SMC hypertrophy and hyperplasia were underlying factors in the observed increase in vascular wall area of uterine arteries during gestation (3, 22). The potential role of relaxin in these pregnancy-associated changes, if any, remains to be determined.
It is possible that the relaxin-mediated increase in wall area is secondary to the increase in renal blood flow observed during exogenous rhRLX administration to males and nonpregnant females (10, 43) or during pregnancy (4). Geometric remodeling of the vascular wall has also been reported in arteries during other physiological and experimentally induced conditions that result in a chronic increase in blood flow. Rat mesenteric arteries subjected to chronically elevated blood flow have been shown to exhibit a time-dependent increase in vascular wall area (45). In another study (2) using a similar animal model, mesenteric arteries subjected to chronically reduced blood flow were characterized by decreased vascular wall area resulting from both the loss and reduction in size of SMCs. Conversely, chronically elevated blood flow in similar arteries yielded increased wall area as a result of increased SMC proliferation (2). It has been suggested that a chronic increase in blood flow results in an increase in wall shear stress, which in turn can stimulate the expression of oncogenes and the production of vasoactive substances and growth factors that contribute to the hypertrophy of the vascular wall (11, 12, 46). Unexpectedly, the geometric remodeling was not observed in all artery types.

**Site-Specific Effects of Relaxin**

Relaxin-induced vascular remodeling appears to be vesselspecific. In contrast to small renal arteries, external iliac arteries isolated from rhRLX-treated or RLX−/− mice did not exhibit any vascular wall remodeling (geometric or compositional) compared with respective controls, and consequently, passive mechanical properties were unchanged as well. There are a couple of possible explanations for these discordant observations. First, if relaxin-induced vascular remodeling is
vascular wall and that as wall strain increases collagen plays a
critical role. It is generally accepted that under conditions of low wall
strain, elastin is the primary load-bearing component of the
arteries. However, at higher levels of circumferential strain in these
arteries, we did not observe any difference in tissue strain
energy between the rhRLX- and vehicle-treated small renal
arteries are. That is, we wanted to know the magnitudes of biaxial
mechanical behavior of dog carotid arteries following treatment with elastase or collagenase. They showed that degradation of collagen had no significant effects on axial stress and reduced circumferential stress when the arteries were pressurized by ≥60 mmHg. This indicates that collagen fibers are load bearing primarily in the circumferential direction and at higher levels of circumferential strain.

Based on these observations, we began to investigate what the physiologic set points for circumferential and axial strains are. That is, we wanted to know the magnitudes of biaxial strains for small renal arteries under normal physiologic loading conditions. Our preliminary data indicate that in situ circumferential and axial Green-Lagrange strains in small renal arteries are ~1.1 and ~0.5, respectively. At these magnitudes of strains, we did not observe any difference in tissue strain energy between the rhRLX- and vehicle-treated small renal arteries (see Fig. 6). Thus, under conditions of normal physiologic circumferential and axial strains, the increased passive compliance observed in these arteries following relaxin treatment (6) is primarily attributable to the relaxin-induced geometric remodeling.

Previous work (24) has shown that there is a 50% increase in the volume of the kidney during human pregnancy, suggesting that small renal arteries may be subjected to significantly higher levels of strain in the circumferential and axial directions in this physiological state. Because endogenous circulating relaxin is known to be significantly elevated during pregnancy, our data suggest that the relaxin-mediated remodeling of small renal arteries during pregnancy would lead to lower vascular wall stresses compared with a situation wherein these arteries had not been remodeled. Thus the relaxin-induced reduction in tissue strain energy and circumferential and coupling tangent moduli at high strains can be considered to be an adaptive response that guards against excessive rise in vascular wall stress due to the kidney enlargement during pregnancy.
In conclusion, relaxin induces both outward geometric (increased unstressed wall area and unstressed wall area-to-lumen area ratio) and compositional (decreased collagen-to-total protein ratio) remodeling in small renal arteries but not external iliac arteries. The relaxin-induced compositional remodeling of small renal arteries becomes functionally relevant only under conditions of high circumferential strains. Thus, under conditions of low-to-physiological circumferential and axial strains, the relaxin-induced increase in passive compliance of small renal arteries is primarily attributable to the relaxin-mediated vascular wall geometric remodeling.

APPENDIX A: PASSIVE MECHANICAL PROPERTIES OF VASCULAR WALL: BIAXIAL CHARACTERIZATION

Small renal and external iliac arteries were isolated from rhRLX-and vehicle-treated control mice and transferred to an isobaric arteriograph where both ends of the artery were mounted on glass microcannulae and suspended in a custom-made sample chamber (Fig. A1). The commercially available isobaric arteriograph system (Living Systems Instrumentation) allows for the measurement of vessel diameter under controlled luminal pressure. The custom-made sample chamber added the capability of axial stretching of the vessel and measurement of axial force. After the residual blood was flushed from the lumen of the artery, the free end was occluded with a suture and tied to a metal hook attached to a force transducer (model 400A; Aurora Scientific). The glass microcannula was attached to a pressure transducer, a pressure servocontroller, and a peristaltic pump. The vessel is held between the metal hook and a movable arm. Sample vessel area ratio and compositional (decreased collagen-to-total protein ratio) remodeling in small renal arteries but not external iliac arteries. The relaxin-induced compositional remodeling of small renal arteries becomes functionally relevant only under conditions of high circumferential strains. Thus, under conditions of low-to-physiological circumferential and axial strains, the relaxin-induced increase in passive compliance of small renal arteries is primarily attributable to the relaxin-mediated vascular wall geometric remodeling.

Under the assumption of negligible shear, the circumferential ($\lambda_a$) and axial ($\lambda_z$) stretches were calculated as:

$$\lambda_a = \frac{R_o}{R_{oo}} \quad (A1)$$

$$\lambda_z = \frac{L}{L_o} \quad (A2)$$

where $R_o$ and $L$ are the vessel outer radius ($=D_o/2$) and length, respectively, in the loaded configuration and $R_{oo}$ and $L_o$ represent the vessel outer radius and axial length, respectively, in the load-free configuration. Circumferential ($\sigma_{oo}$) and axial ($\sigma_{zz}$) Cauchy stresses were calculated from the experimental data as (19):

$$\sigma_{oo} = P \left( \frac{R_{oo}^{\lambda_a^2} - 1}{H_o} \right) \quad (A3)$$

$$\sigma_{zz} = \frac{F}{\pi H_o (2R_{oo} - H_o)} + \frac{\sigma_{oo}}{2} \quad (A4)$$

where $H_o$ represents wall thickness in the load-free configuration and $P$ represents the intraluminal pressure. The second Piola-Kirchhoff stresses, $S_{oo}$ and $S_{zz}$, were defined as:

$$S_{oo} = \frac{\sigma_{oo}}{\lambda_a^2} \quad (A5)$$

$$S_{zz} = \frac{\sigma_{zz}}{\lambda_z^2} \quad (A6)$$

Green-Lagrange strains were calculated as:

$$E_{oo} = \frac{\lambda_a^2 - 1}{2} \quad (A7)$$

$$E_{zz} = \frac{\lambda_z^2 - 1}{2} \quad (A8)$$

where $E_{oo}$ and $E_{zz}$ represent circumferential and axial components of strain, respectively.

Experimentally measured vascular load-deformation behavior was quantified in terms of an orthotropic tissue strain energy function, $\Psi$ (23, 41). Several different tissue strain-energy functions were tested to determine which best described our data (15, 19, 23, 41, 49). We found that the following four-parameter polynomial model, adapted from the seven-parameter polynomial model of Vaishnav et al. (49), best described our data in the least complex way:

$$\psi = C_1 E_{oo}^2 + C_2 E_{oo} E_{zz} + C_3 E_{zz}^2 + C_4 E_{oo}^2 E_{zz} \quad (A9)$$

For a given $\psi$, circumferential and axial second Piola-Kirchhoff stresses can be calculated as:

$$S_{oo}^\psi = \frac{\partial \psi}{\partial E_{oo}} = 2C_1 E_{oo} + C_2 E_{zz} + 2C_4 E_{oo} E_{zz} \quad (A10)$$

$$S_{zz}^\psi = \frac{\partial \psi}{\partial E_{zz}} = 2C_2 E_{oo} + 2C_3 E_{zz} + C_4 E_{oo}^2 \quad (A11)$$

We derived the four constitutive parameters of the tissue strain energy model (Eq. A9) that best-fit each vessel data using the Levenberg-Marquardt algorithm in MATLAB (The MathWorks, Natick, MA). Specifically, the values of the four parameters ($C_1$-$C_4$) were iteratively optimized for each vessel to minimize the sum of squares of differences between Piola-Kirchhoff stresses calculated from experimental data and those calculated from the four-parameter polynomial model.
measurements (Eqs. A5–A6) and from the tissue strain energy function (Eqs. A10–A11). From the optimized parameter values, we computed strain energy at various combinations of axial and circumferential strains for each vessel. In addition, circumferential (\(T_{M_{zz}}\)), axial (\(T_{M_{zz}}\)), and coupling (\(T_{M_{zz}}\) or \(T_{M_{zz}}\)) tangential moduli were calculated as:

\[
T_{M_{zz}} = \frac{\partial S_{zz}^{0}}{\partial E_{zz}} = 2C_1 + 2C_2 E_{zz} \tag{A12}
\]

\[
T_{M_{zz}} = \frac{\partial S_{zz}^{0}}{\partial E_{zz}} = 2C_1 \tag{A13}
\]

\[
T_{M_{zz}} = T_{M_{zz}} = \frac{\partial S_{zz}^{0}}{\partial E_{zz}} = \frac{\partial S_{zz}^{0}}{\partial E_{zz}} = C_2 + 2C_2 E_{zz} \tag{A14}
\]

**APPENDIX B: BIOCHEMICAL AND HISTOLOGICAL MEASUREMENTS**

**Vascular Wall Protein**

Isolated arteries were assayed for total protein content using the Pierce BCA protein assay kit (Rockford, IL) per instructions provided by the manufacturer and using BSA as a standard. Briefly, the arteries were cleaned of extraneous tissue, lyophilized, minced, and digested for 48 h in a pepsin-HCl solution (0.05 g of pepsin in 50 ml of 0.01 N HCl; 100 \(\mu\)l per 1.0 mg of tissue). Following digestion, the solution was vortexed and a 25-\(\mu\)l sample was added to 100 \(\mu\)l of the BCA working reagent. This solution was incubated at 37°C for 30 min and then allowed to cool to room temperature. Absorbance was determined at 550 nm on a spectrophotometer. Relative elastin content was quantified as the ratio of the mass of elastin (\(\mu\)g) to tissue dry weight (mg). Note that elastin content was normalized by tissue dry weight, instead of total protein (as was done for collagen assessment), because there was insufficient artery mass for both elastin and total protein determinations.

**Vascular Tissue Sections**

Isolated external iliac arteries were fixed in 2% paraformaldehyde in PBS at 4°C for 4 h, rinsed thoroughly in PBS, and then cryoprotected in 30% sucrose at 4°C for at least 24 h. The arteries were next embedded in optimum cutting temperature medium and cut into several 6-\(\mu\)m thick sections and collected on Superfrost Plus microscope slides. To obtain cross sections of small renal arteries, an entire kidney was fixed in 2% paraformaldehyde, cryoprotected in sucrose, and then embedded in optimum cutting temperature medium in a configuration that provided cross sections of small renal arteries. The slides were stored at −80°C until use.

**Immunofluorescence Staining**

After rehydration in PBS, each tissue section was permeabilized with 0.1% Triton X-100 for 15 min and rinsed three times with PBS and then five times with 0.5% BSA in PBS. The sections were next treated with blocking solution (2% BSA in PBS) for 1 h at room temperature followed by another five washes with 0.5% BSA in PBS. They were then incubated with a primary antibody for smooth muscle actin (catalog no. A1978; Sigma-Aldrich, St. Louis, MO) at room temperature followed by an additional five washes with 0.5% BSA in PBS. Next, the tissue sections were stained with a FITC-conjugated secondary antibody for another hour (Sigma-Aldrich) at room temperature followed by five washes with 0.5% BSA and PBS and then five more washes with PBS. Some samples were incubated with a Cy3-conjugated smooth muscle actin antibody (catalog no. C6198; Sigma-Aldrich). Finally, the nuclei were stained with Hoechst dye (Sigma-Aldrich) for 30 s followed by two washes with PBS. Last, the slides were coverslipped with gelvatol mounting media and stored at 4°C in the dark. The tissue sections were imaged with an Olympus Provis fluorescence microscope.

**SMC Density**

Fluorescent images were analyzed using ImageJ software (Wayne Rasband, National Institutes of Health). Figure 1 is a representative image of a small renal artery cross section stained for smooth muscle actin and nuclei. The media of the arterial wall was identified by smooth muscle actin (red) immunofluorescence, and nuclei were stained with Hoechst appeared blue. Nuclei belonging to SMC were designated as those that lay within the smooth muscle actin-positive region of the arterial wall. SMC density for each arterial cross section was quantified as the ratio of number of SMC to arterial media area. The mean SMC density of each artery was assessed by analyzing at least four consecutive cross sections of that artery.

**Vascular Gelatinase Activity**

Vascular gelatinase activity was measured by gelatin zymography as previously described with minor modifications (25–27, 31). Briefly, artery samples 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 \(\mu\)l Protease Inhibitor Cocktail Set III (EMD Chemicals, Gibbstown, NJ) per milliliter of buffer were added to the tissue, which was then homogenized in the WIG-L-BUG. The homogenate was sonicated for 1–2 s on setting 4 (Misonix 2000; 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 micrograms of protein were combined with an equal volume of 2× Novex Tris-glycine SDS sample buffer and electrophoresed on Novex 10% Zymogram (gelatin-containing) Gels for ~2 h 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 ~18 h. 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 h, 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).

ACKNOWLEDGMENTS

We thank Dr. Laura J. Parry (University of Melbourne) for providing the wild-type and relaxin knockout mice. Portions of this work have been presented in abstract form (Debrah DO, Debrah JE, Shroff SG, Conrad KP. Relaxin regulates arterial wall structure and composition. Reprod Sci 15, Suppl 1: 217A, 2008).

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