Aortic stiffness is associated with vascular calcification and remodeling in a chronic kidney disease rat model

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Ng K, Hildreth CM, Phillips JK, Avolio AP. Aortic stiffness is associated with vascular calcification and remodeling in a chronic kidney disease rat model. *Am J Physiol Renal Physiol* 300: F1431–F1436, 2011. First published April 6, 2011; doi:10.1152/ajprenal.00079.2011.—Increased aortic pulse-wave velocity (PWV), an indicator of arterial stiffness, is strongly associated with vascular calcification and remodeling in a chronic kidney disease rat model. *Am J Physiol Renal Physiol* 300: F1431–F1436, 2011. First published April 6, 2011; doi:10.1152/ajprenal.00079.2011.—Increased aortic pulse-wave velocity (PWV) reflects increased arterial stiffness and is a strong predictor of cardiovascular risk in chronic kidney disease (CKD). We examined functional and structural correlations among PWV, aortic calcification, and vascular remodeling in a rodent model of CKD, the Lewis polycystic kidney (LPK) rat. Hemodynamic parameters and beat-to-beat aortic PWV were recorded in urethane-anesthetized animals (12-wk-old hypertensive female LPK rats (n = 5)) before the onset of end-stage renal disease and their age- and sex-matched normotensive controls (Lewis, n = 6). Animals were euthanized, and the aorta was collected to measure calcium content by atomic absorption spectrophotometry. A separate cohort of animals (n = 5/group) were anesthetized with pentobarbitone sodium and pressure perfused with formalin, and the aorta was collected for histomorphometry, which allowed calculation of aortic wall thickness, medial cross-sectional area (MCSA), elastic modulus (EM), and wall stress (WS), size and density of smooth muscle nuclei, and relative content of lamellae, interlamellae elastin, and collagen. Mean arterial pressure (MAP) and PWV were significantly greater in the LPK compared with Lewis (72 and 33%, respectively) animals. The LPK group had 6.8-fold greater aortic calcification, 38% greater aortic MCSA, 56% greater EM/WS, 13% greater aortic wall thickness, 21% smaller smooth muscle cell area, and 20% less elastin density with no difference in collagen fiber density. These findings demonstrate vascular remodeling and increased calcification with a functional increase in PWV and therefore aortic stiffness in hypertensive LPK rats.

hypertension; arterial stiffness; pulse-wave velocity; histomorphometry; elastin; collagen; hypertrophy; aorta; calcium

INCREASED AORTIC PULSE-WAVE velocity (PWV), an indicator of arterial stiffness, is a strong and independent predictor of cardiovascular risk in both hypertensive and chronic kidney disease (CKD) patients (7, 30, 39, 44). Patients with CKD have increased morbidity and mortality due to cardiovascular disease than to end-stage renal disease (ESRD) (15, 20). The high incidence of cardiovascular disease in CKD could be due to a loss of large artery function and the capacity to buffer pulsatile changes in blood pressure (BP) associated with vascular stiffness. A distensible aorta ensures a steady flow of blood to end organs with reduced exposure to high systolic BP (SBP). In both humans (28, 35, 38) and rats (12), a loss of aortic distensibility results in a stiff conduit that is less able to absorb cardiac afterload. This results in increased pulse pressure (PP) and associated cardiovascular risk.

Adaptation to higher BP (45) or aortic calcification (27, 42) results in the reduction of the elastic nonuniformity of the aortic trunk with an increase in stiffness in the abdominal aorta causing a reduction in impedance mismatch. This results in a uniform stiffness of the aortic trunk, with a more rapid return of reflected waves from the distal vasculature, which further augments SBP (27). Regardless of the underlying mechanism, a loss of large artery function also results in a reduced capacity of the system to buffer pulsatile changes in BP, with vulnerable end organs at risk of microvascular damage, particularly in the kidney and brain (36). Microvasculature damage results in altered capillary proliferation, increased thrombosis, and vaso-motor dysfunction (8), all of which are characteristic features of aging (2). In addition, capillary rarefaction in peripheral beds could further increase peripheral resistance, contributing to hypertension and arterial stiffness (3), as well as being related to efficacy of antihypertensive treatment (14). This accelerated aging process is evident in patients starting dialysis (11, 18), and in ESRD a cyclic situation exists where a reduction in renal function increases arterial stiffness and cardiovascular risk (40), which in turn contributes to the progression of renal disease. Pathological vascular changes are likely to occur early in the course of renal disease. For example, in young normotensive autosomal dominant polycystic kidney disease (ADPKD) patients, where renal function is preserved, pulse-wave reflection is amplified (9).

Arterial stiffness can be measured via a number of different approaches. PWV is often used as a surrogate measure of arterial stiffness and has been used in several large-scale clinical trials on mortality and cardiovascular events in ESRD (7, 40), hypertensive subjects (27, 29, 43), the elderly (27), subjects with metabolic disorders (40), and the general population (27). Blacher et al. (7) found that an increase of 1 m/s in PWV increased all-cause mortality by 39%. Arterial stiffness is mainly affected by age-related alterations in arterial wall structure and geometry and is dependent on BP. Aortic stiffness is also associated with aortic calcification in humans (27). Experimental studies involving hypertension and calcification have shown that PWV is also increased in spontaneously hypertensive rats (SHR) (12), vitamin D₃- and nicotine-calcified rats (21, 33, 34), and rats with renovascular hypertension (22).

We have recently established a rodent model of CKD in the form of autosomal recessive (AR) PKD. (37). The Lewis polycystic kidney (LPK) rat developed from a spontaneous mutation and demonstrates hypertension by 6 wk of age, initial signs of renal dysfunction at 12 wk of age, and progress to ESRD by 18–24 wk of age, concurrent with increased cardiac mass and left ventricular hypertrophy (LVH) (37). At 12–13 wk of age, when hypertension is well established yet renal function is not significantly impaired, the LPK rats have
increased sympathetic control of the heart and vasculature (16), presumably contributing to the hypertensive state and therefore development of cardiovascular disease. Given the association between hypertension, CKD, and increased arterial stiffness (9, 21, 44), it is possible that the LPK rats have increased arterial stiffness as a result of arterial calcification and changes in vascular wall structure, which may also contribute to the development and/or maintenance of hypertension and therefore cardiovascular disease. We have therefore examined the hypothesis that as a result of their hypertensive status, the LPK rats demonstrate pathological markers of arterial stiffness, such as vascular calcification and morphological changes in the structure of the aorta, which correlate with a functional increase in PWV and PP, factors which in turn drive higher cardiovascular risk and end-organ damage.

MATERIALS AND METHODS

Animals. All experiments were approved by the Macquarie University Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals, as endorsed by the National Health and Medical Research Council of Australia. Female LPK (n = 10 total, 12 wk old) and age-matched female Lewis (n = 11 total) rats were used.

Hemodynamic measurements. Five LPK and six Lewis rats were anesthetized with urethane (ethyl carbamate, 1.3 g/kg). The depth of anesthesia was assessed regularly by assessing the reflex response to noxious stimuli (hindpaw pinch) or tactile stimuli (corneal stroking). Two 1.2-F, high-fidelity, pressure sensor catheters (Millar SPC-835) were introduced via the carotid and femoral arteries into the descending aortic trunk. PWV in the aortic segment between the two sensors was calculated using the foot-to-foot method (31, 32), the foot being defined by the peak of the second time derivative of pressure during each pulse. PWV of the whole aortic segment was measured with sensors located at 8 cm apart.

MAP was raised and lowered to obtain a full physiological range of BP using intravenous infusion of phenylephrine (50 μg/min) and sodium nitroprusside (10 μg/min), respectively. Data were acquired at a sampling rate of 2 kHz (Power 1401, Cambridge Electronic Design), and PWV was calculated online using custom-written scripts using Spike 2 software (version 7.02, Cambridge Electronic Design). MAP was recorded and corresponding PWV was calculated for each individual pulse during the experiment. PWV was plotted against MAP to construct phase plots to characterize PWV over a wide range of MAP from 60 to 200 mmHg in the aorta. The average total duration of the experiment was 2 h from induction of anesthesia, and PWV was measured over a 30-min period.

Arterial calcification. Following the hemodynamic measurements described above, each rat was euthanized with KCl (3 M) and the aorta was harvested, weighed, and heated to constant dry weight. Calcium content was determined using atomic absorption spectrophotometry as described previously (21, 33, 34). Briefly, dry samples were dissolved in nitric acid (14 N, 72 h, room temperature). The hydrolysate was centrifuged at 2,000 g for 10 min before the supernatant was removed. Lanthane chloride was added, and the supernatant was atomized. Total calcium values were expressed as micrograms per gram tissue dry weight derived from the absorbance measured.

Histomorphometry. Five LPK and five Lewis rats were anesthetized with pentobarbitone sodium (60 mg/kg ip). Each animal was perfused at 110 mmHg with 0.9% saline followed by 4% formalin containing phosphate-buffered saline for 45 min. A 3-mm sample of the thoracic aorta was excised and preserved in 4% formalin for histological study. Four-micrometer sections of aorta were stained with Martius/scarlet/blue stain to identify collagen deposition and smooth muscle cell nuclei, or Shikata’s orcein stain to identify the lamellae and interlamellae elastin. Histomorphometric analysis was performed using a customized automated image-processing software, Image J (1). Images were acquired using a video camera mounted on a microscope (Zeiss Z1, Gottingen, Germany) and processed with Zeiss Axiovision software (Zeiss Z1). Analog images were digitized and compared by setting a minimum threshold that allowed for visualization of elastin, collagen, and smooth muscle cells. These images were then binarized to extract relative measures of elastin, collagen, and smooth muscle cells across the vessel wall. The medial elastin network was defined as the relative area occupied by elastin lamellae and interlamellae elastin, the mean thickness of each elastin lamella, and the mean interlamellae distance. The measurements and calculations were made in four to six fields of interest equally distributed around the circumference from each section. The elastin-to-collagen ratio was defined as the ratio of their respective densities to the surface of the studied field. Nuclei were automatically counted by the software, without the counting of any holes or borderline nuclei. Nuclei density was defined as the number of nuclei per unit surface area. Quality control on the binarized images was done by visual inspection and comparison with the original image to evaluate the integrity of the customized automated software by two investigators.

The measurement of medial cross sectional area (MCSA), elastic modulus (EM), and wall stress (WS), and aortic geometry were calculated assuming circular structure

\[ \text{MCSA} = \frac{\pi}{4} (D_o^2 - D_i^2) \]  

where \( D_o \) and \( D_i \) are the outer and inner diameters (in mm).

EM and WS (10⁶ dyne/cm²) were calculated from the Moens-Korteweg and Lamé equation

\[ \text{EM} = \left( \frac{\text{PWV}^2 \cdot D_i}{h} \right) \]  
\[ \text{WS} = \left( \frac{\text{MAP} \cdot D_i}{2h} \right) \]

where \( p \) is the density of blood (1.05 g/cm³), and \( h \) is the wall thickness. MCSA is not influenced by blood or fixation pressures because of the intrinsic assumption used in the equation that vessel walls are incompressible.

Statistical analysis. All values were averaged and expressed as means ± SE unless stated otherwise. Unpaired Student’s t-tests were performed to compare LPK and Lewis controls. PWV were compared between LPK and Lewis rats across a common MAP range (60–200 mmHg) using two-way ANOVA. Second-order polynomials were fitted to PWV-MAP curves over this range of MAP based on goodness of fit. \( P < 0.05 \) was considered significant.

RESULTS

Hemodynamic measurements. The LPK rats displayed a phenotypic elevation in MAP, SBP, PP, and HR compared with Lewis rats (Table 1) as described previously (16, 37). Body weight and length measured between the sternum and tail-femoral bifurcation were not different between the groups.

PWV was measured from 60 to 200 mmHg in both LPK and Lewis rats. In the LPK rats, PWV was elevated across the whole pressure range (\( P < 0.001 \), Fig. 1). Mean values of PWV as a function of MAP were described by fitting a quadratic polynomial for the full range of experimental pressure of each strain. The nonlinear fit for the LPK rat was shifted upward on average across the whole MAP range (Fig. 1). The coefficients of these polynomials are shown in Table 2. These
groups (see Table 2). A relationship exists between MAP and PWV in both the Lewis and LPK rat upward due to higher stiffness of the aorta in the LPK rats. A nonlinear kidney disease (CKD) on isobaric characteristic of PWV. The curve is shifted SBP, mmHg 141
Body length, cm 11
SBP, mmHg 141 ± 7
238 ± 17 <0.001
MAP, mmHg 108 ± 3
191 ± 14 <0.001
DBP, mmHg 87 ± 3
139 ± 14 0.005
PP, mmHg 54 ± 5
98 ± 11 0.005
HR, beats/min 297 ± 28
376 ± 11 0.041
Values are means ± SE; n = 6 for Lewis and 5 for Lewis polycystic kidney (LPK) rats. SBP, systolic blood pressure; MAP, mean arterial pressure; DBP, diastolic pressure; PP, pulse pressure; HR, heart rate; NS, difference not significant.
polynomials show an offset of 0.98 m/s for LPK compared with Lewis rats.

Structural differences in the descending aorta of LPK compared with Lewis rats. The structural properties of the descending aorta were examined through quantifying calcium content, collagen deposition, smooth muscle cell nuclei density, and lamellae and interlamellae elastin thickness.

In the LPK, total calcium content in the aorta was 6.5-fold greater compared with Lewis rats (Fig. 2). Figure 3 shows a typical example of a longitudinal section of the aortic wall, stained with Shitaka’s orcein and Martius/scarlet/blue in the LPK and Lewis rat. Table 3 shows the group data for each of the vascular parameters examined. The LPK rats exhibited 12% greater medial thickness, indicating medial hypertrophy. LPK rats also showed a 56% greater EM/WS ratio. An increase in EM/WS indicates relative stiffening. In the LPK rats, smooth muscle cell nuclei density was 27% greater. Additionally, there was a 30 and 13% less lamellae elastin density and interlamellae elastin density, respectively, in the LPK compared with Lewis rats. The thickness of elastin lamellae was 11% less in LPK compared with Lewis rats, and the elastin-to-collagen ratio was also reduced by 21% in LPK relative to Lewis rats. Morphometric measurements of nuclei revealed that the cross-sectional area of nuclei was 21% larger in the Lewis rats. After normalization for wall stress, the relative elastic modulus of LPK was 56% higher compared with Lewis rats.

**DISCUSSION**

The major finding in the present study is that LPK rats exhibit increased isobaric arterial stiffness, as indicated by a higher PWV, PP, and an increased EM/WS ratio compared with Lewis controls. This increase in PWV is associated with increased arterial calcification, medial thickness, and cross sectional area, consistent with our hypothesis that this hypertensive model would demonstrate pathological markers of arterial stiffness.

Intrinsic changes in the artery wall which lead to increased aortic stiffness at a particular pressure determine increased PWV (23, 30). The LPK rats exhibited increased PWV independently of resting BP, in that at all BPs studied the LPK rats had a higher PWV. This pressure-independent increase in PWV may be due to structural abnormalities in the LPK rat, consistent with a decrease in elastin content and the elastin-to-collagen ratio, and therefore a relative increase in collagen which will promote arterial stiffness. In CKD patients, PWV is similarly increased; however, whether the increase in PWV is independent of BP is not as readily determined, as PWV is not calculated over a large BP range in human subjects (25), as has been done in this study. Nonetheless, as high BP is significantly correlated with increased PWV in CKD and renal transplantation reduces PWV independently of resting BP (19),

Table 1. Basal hemodynamic parameters in Lewis and LPK rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lewis</th>
<th>LPK</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>183 ± 1</td>
<td>181 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Body length, cm</td>
<td>11 ± 0.5</td>
<td>12 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>141 ± 7</td>
<td>238 ± 17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>108 ± 3</td>
<td>191 ± 14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>87 ± 3</td>
<td>139 ± 14</td>
<td>0.005</td>
</tr>
<tr>
<td>PP, mmHg</td>
<td>54 ± 5</td>
<td>98 ± 11</td>
<td>0.005</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>297 ± 28</td>
<td>376 ± 11</td>
<td>0.041</td>
</tr>
</tbody>
</table>

A second-order polynomial equation was fitted to pulse-wave velocity (PWV)-MAP curve of each strain over MAP between 60 and 200 mmHg. a, b, and c: Regression coefficients such that PWV = aMAP^2 + bMAP + c.

Fig. 1. Pulse-wave velocity (PWV)-mean arterial pressure (MAP) curves are averaged over 5-mmHg pressure steps in Lewis (○) and Lewis polycystic kidney (LPK) rats (●). Values are means ± SE. Results show effect of chronic kidney disease (CKD) on isobaric characteristic of PWV. The curve is shifted upward due to higher stiffness of the aorta in the LPK rats. A nonlinear relationship exists between MAP and PWV in both the Lewis and LPK rat groups (see Table 2).

Fig. 2. Aortic calcium content of LPK rats showed a 6.5-fold increase compared with Lewis rats (Lewis: 66.8 ± 5.7 μmol/g; LPK: 434.0 ± 118.3 μmol/g, P < 0.001). This increase in calcium content is associated with an isobaric increase in PWV and structural changes in both elastin and collagen fibers as determined by histomorphometry.
it would suggest that increased PWV occurs in CKD as a consequence of kidney disease, and may be a contributing factor to the maintenance of hypertension.

In the LPK rats, there was a marked elevation in aortic calcification. Increased arterial calcification is widely documented in patients with CKD and ESRD and may be a contributing factor to their increased risk of cardiovascular disease (5, 26, 41). Pathologically, increased arterial calcification leads to a decrease in wall elasticity by reducing the amount of low elastic modulus protein (elastin and smooth muscle) and/or increase in high elastic modulus protein (collagen) and therefore arterial stiffening (21, 33, 34). The underlying mechanism resulting in calcification in CKD/ESRD is not clear. It may be triggered by uremia, acting as a key stimulus to vascular hypertrophy (24). Other factors are also likely to be involved; for example, an accumulation of hyaluronan in the aortic media results in alterations in collagen and elastin interstitial composition, which leads to an increase in arterial stiffness (10).

In the LPK rats, the lamellae and interlamellae elastin content in the wall of the descending aorta was reduced. This contrasts with previous work conducted in experimental renal failure. In the subtotally nephrectomized rat, aortic wall thickness, cross-sectional media, total number of aortic vascular smooth muscle cells, and volume of extracellular matrix including collagen were significantly increased, whereas cellular hypertrophy was only modest and an increase in elastic fibers did not occur (4). However, BP was not elevated in this rat model, whereas in the LPK rats BP is markedly elevated. This may account in part for these differences. Nevertheless, in both the LPK and subtotally nephrectomized rats, the elastin-to-collagen ratio was reduced, indicating an increase in arterial stiffness in both models of renal disease. One other contributor to increased arterial stiffness in the LPK rats is an increase in nucleus density, which may arise from an increase in DNA content or polyploidy (24). An increase in DNA content has been proposed to cause an increase in interstitial matrix synthesis by smooth muscle cells, leading to vascular hypertrophy (24).

Table 3. Morphometric parameters of the descending thoracic aorta

<table>
<thead>
<tr>
<th>Morphological Parameter</th>
<th>Lewis</th>
<th>LPK</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal diameter, mm</td>
<td>1.177 ± 0.016</td>
<td>1.398 ± 0.003</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>External diameter, mm</td>
<td>1.312 ± 0.013</td>
<td>1.551 ± 0.024</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Medial thickness, μm</td>
<td>67.5 ± 10.7</td>
<td>76.5 ± 2.6</td>
<td>0.024</td>
</tr>
<tr>
<td>Medial cross-sectional area, mm²</td>
<td>0.26 ± 0.05</td>
<td>0.36 ± 0.02</td>
<td>0.018</td>
</tr>
<tr>
<td>Elastic modulus/wall stress</td>
<td>1.47 ± 0.03</td>
<td>2.29 ± 0.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total elastin density, %</td>
<td>63 ± 8</td>
<td>51 ± 10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of lamellae</td>
<td>8 ± 1</td>
<td>8 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Lamellae elastin density, %</td>
<td>24 ± 5</td>
<td>17 ± 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interlamellae elastin density, %</td>
<td>39 ± 7</td>
<td>34 ± 9</td>
<td>0.024</td>
</tr>
<tr>
<td>Thickness of elastin lamellae, μm</td>
<td>3.13 ± 0.57</td>
<td>2.80 ± 0.44</td>
<td>0.021</td>
</tr>
<tr>
<td>Lamellae-to-interlamellae elastin ratio</td>
<td>0.64 ± 0.21</td>
<td>0.55 ± 0.23</td>
<td>NS</td>
</tr>
<tr>
<td>Total collagen density, %</td>
<td>26 ± 9</td>
<td>30 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Elastin-to-collagen ratio</td>
<td>2.75 ± 1.3</td>
<td>1.75 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Area, μm²</td>
<td>14 ± 4</td>
<td>11 ± 2</td>
<td>0.017</td>
</tr>
<tr>
<td>Nucleus density, %</td>
<td>0.15 ± 0.06</td>
<td>0.19 ± 0.04</td>
<td>0.041</td>
</tr>
</tbody>
</table>

Values are means ± SD. Each parameter is an average of 4–6 fields equally distributed around the circumference of the aorta from each segment of thoracic aorta. P < 0.05 is considered significant.

Fig. 3. Typical histological sections stained with Shitaka’s orcein (top) and Martius/scarlet/blue (bottom). Shown is a longitudinal section of the thoracic descending aorta in a Lewis (left) and LPK (right) rat. A reduction in lamellae elastin density and interlamellae elastin density can be observed in the LPK compared with Lewis rat. The average thickness of elastin lamellae was reduced in the LPK rat. The cross-sectional area of nuclei was noticeably smaller in the LPK compared with Lewis rat. Morphometric measurements of nuclei revealed that the cross-sectional area of nuclei was 21% larger in the Lewis rat.
various uremia-specific proinflammatory factors are upregulated, which in turn promote expansion of the lesions, further accelerating calcification and therefore increasing arterial stiffness in LPK rats (13, 17).

In summary, we have demonstrated that LPK rats develop arterial stiffness, aortic calcification, and structural changes in the aortic vascular wall by 12 wk of age, indicating likely arterial stiffness, aortic calcification, and structural changes in LPK rats (13, 17).

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