Angiotensin-converting enzyme inhibitor limits pulse-wave velocity and aortic calcification in a rat model of cystic renal disease

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Angiotensin-converting enzyme inhibitor limits pulse-wave velocity and aortic calcification in a rat model of cystic renal disease.

Pulse-wave velocity (PWV) is a well-established measure of arterial stiffness that has gained acceptance as an index of cardiovascular risk prediction in high-risk patients with end-stage renal disease (ESRD) and is also proving to be a useful predictor of response to antihypertensive therapy (19, 44). Under normal conditions, a distensible aorta ensures a steady flow of blood to end organs with reduced exposure to high systolic blood pressure (SBP). In both humans and the rat, a loss of aortic distensibility results in a stiff conduit that is less able to buffer pulsatile changes in blood pressure, increasing both SBP and pulse pressure, placing vulnerable tissues at risk of microvascular damage, particularly the kidney and brain (15, 32, 33, 36). This is evident in patients starting dialysis and in ESRD, where a cyclic situation exists such that a reduction in renal function increases arterial stiffness and blood pressure, which in turn contributes to the progression of renal disease (12, 13, 20, 41).

In CKD, the remodeling that occurs is comparable to the arteriosclerosis of aging, characterized by aortic wall stiffness due to extracellular matrix remodeling of the media, with calcification and breakdown of elastic and collagen fiber structure (26, 34, 44). This is quite distinct from the obstructive and thrombogenic atherosclerotic condition, where calcification is predominantly intimal, associated with lipid and cholesterol plaques (4). There is no specific treatment for arteriosclerosis, and research for drug targets is limited by lack of appropriate animal models (24, 40).

Current approaches to the treatment of hypertension in CKD involve aggressive control of blood pressure, often with inhibitors of the renin-angiotensin system (RAS), either angiotensin-converting enzyme (ACE) inhibitors or ANG II receptor-blocking agents (16). The ability of these drugs to attenuate or reverse vascular remodeling in conduit vessels, in both humans and animal models of disease, is consistent with the growth-promoting, prooxidative and proinflammatory actions of ANG II (11, 25, 37, 38). The capacity of ACE inhibitors to limit aortic calcification has been demonstrated in an induced model of arterial calcification, the vitamin D₃ and nicotine rat (42). What is not clear, however, is the impact of RAS inhibition on arterial calcification in a more complex disease state, such as CKD, and critically, if any response to ANG II inhibition can be related to arterial changes, as determined by both PWV and the structural indices of vascular remodeling associated with kidney disease.

We have demonstrated that the Lewis polycystic kidney (LPK) rat, an autosomal recessive model of cystic renal disease, exhibits features of arteriosclerosis, including increased functional aortic stiffness, vascular remodeling, and calcification at 12 wk of age, a time point where hypertension is developed and rats are on the verge of progression to renal...
failure (30). The aim of the present study therefore was to examine the hypothesis that perindopril, a commonly used ACE inhibitor, would limit the development of vascular remodeling and aortic calcification in the LPK rat and that this would be associated with a blood pressure-independent reduction in functional stiffness.

MATERIALS AND METHODS

Animals

Animals were obtained from the Animal Resources Centre, Perth, Western Australia, Australia. Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals, and the study was approved by the Macquarie University Animal Ethics Committee. Experiments were performed in three groups of age-matched LPK and control Lewis rats with an equal number of each gender designated to each group. The first group of 6-wk-old animals (untreated) were assessed for PWV (n = 6/group). The second and third groups of animals were randomly assigned to receive either no treatment (untreated) or perindopril (3 mg·kg⁻¹·day⁻¹, Servier Laboratories, Australia) in their drinking water from 6 to 12 wk of age (n = 6/group) (3, 42). Animals were weighed, and SBP was measured using tail-cuff plethysmography (ITC Life Science) from 6 wk of age before drug treatment and then weekly until 11 wk. Perindopril was stopped 1 day before determination of PWV parameters at 12 wk of age. In total, 36 rats were used. All animals were housed in a temperature-controlled room with a 12:12-h day-night cycle. Food and water were available ad libitum.

PWV

Animals were anesthetized with ethyl carbamate (urethane; 1.3 g/kg ip, Sigma-Aldrich). The right jugular vein and the left femoral vein were cannulated for the administration of phenylephrine and sodium nitroprusside, respectively. Intra-arterial blood pressure and PWV were recorded using two high-fidelity 1.2F catheter-tipped pressure sensors implanted via the carotid and femoral arteries, positioned at the aortic arch and abdominal aorta just proximal to the iliac bifurcation, respectively. Mean arterial pressure (MAP) was determined from measurements made by the proximal pressure transducer located in the aortic arch. Arterial pressure was increased and decreased by infusion of phenylephrine (50 µg/min) and sodium nitroprusside (10 µg/min), respectively. The actual distance between the two sensors was determined post mortem. PWV was calculated by dividing the propagation distance by propagation time in the units of meters per second using an automated online foot-to-foot method. Waveforms were differentiated twice to define the location of the foot of the pressure pulse. PWV-MAP phase plots were obtained within the pressure range of 60–200 mmHg. All calculations were done using Spike2 v.6, software (Cambridge Electronic Design, Cambridge, UK). PWV was measured over a 30-min period.

Histomorphometry

Following PWV measurements and euthanasia, animals were perfused at 110 mmHg with 0.9% saline followed by 4% formalin containing 0.1 M phosphate-buffered saline (pH 7.4) for 45 min. A 3-mm sample of the descending thoracic aorta was excised and postfixed overnight in the same solution at 4°C. Four-micrometer paraffin-embedded sections of aorta were subsequently cut and stained with martius/scarlet/blue stain to identify collagen deposition and smooth muscle cell nuclei, and 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), processed with Zeiss Axiosvision software (Zeiss Z1). Analog images were digitized and compared by setting a minimum threshold that allowed for visualization of elastin, collagen, or smooth muscle cells only. The relative area occupied by elastin lamellae and interlamellae elastin, mean thickness of each elastin lamella, mean interlamellae distance, and collagen content was obtained. The measurements and calculations were made in six fields in each section equally distributed around the circumference from each section. The elastin-to-collagen ratio (E/C) was defined as the ratio of their respective densities to the surface of the studied field. Binarized images were put side-by-side with the original images to compare and evaluate the integrity of the customized automated software by two investigators.

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

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

where \(D_o\) and \(D_i\) are the outer and inner diameters (in mm). EM and WS (10⁶ dyne·cm⁻²) were calculated from the Moens-Korteweg and Lame equations as follows

\[
\text{EM} = \frac{\text{PWV}^2 \cdot D_o \cdot \rho}{h}
\]

\[
\text{WS} = \frac{D_i \cdot \text{MAP}}{2h}
\]

where \(\rho\) 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.

Total calcium levels. The remaining aorta left from histology was weighed and heated to constant dry weight. Calcium content was determined using atomic absorption spectrophotometry as described previously (31). 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 micromoles per gram tissue dry weight derived from the absorbance measured.

Statistical Analysis

Results are presented as means ± SE except in determining morphometric density parameters, where means ± SD data are presented. Preliminary analysis revealed there was no statistical difference between males and females for all parameters measured with the exception of body weight, and data were therefore grouped for statistical comparison. Strain and drug treatment effects were determined using a one-way ANOVA with Tukey’s post hoc analysis for multiple comparisons. For SBP as determined by tail cuff, the 6-wk age group data were analyzed as a control independently of the subsequent weekly blood pressure determinations, as it was measured before initiation of therapy. To determine the effect of strain and age on the effect of drug treatment on tail-cuff SBP, two-way ANOVA for repeated measures and Bonferroni’s correction were used to determine statistical significance between specific time points.

RESULTS

Body Weight and Tail-Cuff SBP

Body weight increased in all animals from 6 to 12 wk of age. At 12 wk of age, female rats had lower body weight compared with males and Lewis control rats were heavier than LPK rats ($P < 0.001$, Lewis male: 281 ± 15 g, Lewis female: 174 ± 5 g; LPK male: 244 ± 42 g, LPK female: 139 ± 12 g). There

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was no effect of treatment with perindopril on body weight for either strain (Lewis: $P = 0.83$; LPK: $P = 0.59$).

At 6 wk of age, before administration of perindopril, there was no difference in tail-cuff SBP between treated or untreated animals within each strain; however, the LPK animals demonstrated higher tail-cuff SBP compared with the Lewis control rats ($142.2 \pm 5.5$ vs. $107.9 \pm 4.5$ mmHg, respectively, $P < 0.001$, $n = 24$).

Over the ages of 6–11 wk, there was no change in the tail-cuff SBP of the Lewis animals (Fig. 1A), while in the LPK rats, blood pressure increased between weeks 6 and 7 ($P < 0.05$) and remained elevated to week 11 (Fig. 1B). In both the Lewis control and LPK rats, perindopril reduced tail-cuff SBP over the treatment period ($P < 0.05$). In the Lewis rats, this effect of perindopril was most apparent at ages 8 and 10 wk (Fig. 1A). In the LPK rats, perindopril treatment prevented the age-related increase in tail-cuff SBP that occurred from 7 wk onward (Fig. 1B), and SBP was reduced compared with untreated LPK at all time points ($P < 0.05$). Tail-cuff SBP in the treated LPK rats remained higher than in either of the Lewis groups ($P < 0.05$).

**Isobaric PWV**

Figure 2 shows PWV within the pressure range of 60–200 mmHg for each group of rats. The LPK has higher PWV compared with Lewis across a physiological range of MAPs (80–150 mmHg) at both 6 and 12 wk of age ($P < 0.05$), and for both the Lewis and LPK isobaric PWV increased between 6 and 12 wk of age ($P < 0.05$). Perindopril had no impact on PWV in the Lewis animals. In contrast, perindopril treatment of the LPK rats caused a regression (downward and to the left) in PWV variables ($P < 0.05$) to levels that were not significantly different from that of the 6-wk-old LPK and 12-wk-old Lewis rats. Isobaric PWV of the LPK rats showed a reduction of at least 18% ($P < 0.05$) after ACEi.

**Aortic Calcification**

As illustrated in Fig. 3, 12-wk-old untreated LPK showed sixfold higher levels of aortic calcification compared with age-matched Lewis controls (Fig. 3, $P < 0.001$). There was also a noticeable trend for higher calcium levels in the 6-wk-old LPK compared with age-matched controls (LPK 6 wk 123.1 ± 8 vs. 65.5 ± 3 μmol/g, $P = 0.056$). Treatment with perindopril did not alter aortic calcification in the Lewis rats but did cause a significant reduction in the aortic calcification in the LPK rats ($P < 0.001$), resulting in calcium levels comparable to that of the 6-wk-old LPK ($P = 0.153$), but still greater than that of the Lewis rats from either group ($P < 0.001$).

**Arterial Wall Properties**

**MCSA and EM/WS.** MCSA in the Lewis animals did not differ between 6 and 12 wk of age and was not influenced by perindopril treatment (Fig. 4). In the LPK rats, MCSA was greater than in the Lewis control animals at both 6 and 12 wk of age and showed an age-related increase over this time period (Fig. 4; $P < 0.05$). Treatment with perindopril prevented this age-related hypertrophy, with MCSA values in the 12-wk-old treated LPK rats staying at levels comparable to the 6-wk-old LPK rats ($P = 0.886$). EM/WS was greater in the LPK compared with age-matched Lewis controls ($P < 0.01$). Perindopril prevented an age-related increase in EM/WS in the LPK ($P < 0.001$), but not Lewis rats (Fig. 5).

**Vessel wall composition.** The elastin and collagen features of the descending thoracic aorta of all groups of rats are summarized in Table 1. At 6 wk of age, the LPK rats had 21% less total elastin density compared with age-matched Lewis animals. This difference in elastin content was due to reduced lamellae elastin density ($P < 0.001$), but not lamellae elastin number or interlamellae density (Table 1). At 12 wk, there was a trend toward a significantly decreased total elastin density in untreated LPK compared with untreated Lewis rats, but this was not significant ($P = 0.077$). Treatment with perindopril caused an increase in the total elastin in the LPK rats at 12 wk ($P < 0.01$; Fig. 6). Total collagen density was not different in 6- or 12-wk-old LPK compared with age-matched Lewis rats; however, perindopril treatment reduced total collagen density by 22% in LPK rats at 12 wk compared with untreated animals ($P < 0.05$; Fig. 6). Overall, these changes were reflected by a

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reduced E/C in 6- and 12-wk-old LPK rats compared with Lewis age-matched controls ($P < 0.01$) and a trend toward more compliant arteries (increased E/C) in the perindopril-treated 12-wk-old LPK rats, although this did not reach statistical significance compared with untreated 12-wk-old LPK rats ($P = 0.089$).

**DISCUSSION**

We have demonstrated that treatment with the ACE inhibitor perindopril prevents an increase in isobaric PWV, a blood pressure-independent measure of arterial stiffness, in parallel with preservation of aortic structural integrity and...
inhibition of aortic calcification. Our analysis of functional and structural indices of aortic stiffness, and the response to intervention, suggests a hitherto undescribed capacity for the RAS to drive arteriosclerosis in a cystic renal disease model.

By undertaking a longitudinal study in the LPK animal model, we have been able to extend our previous work, showing that intrinsic wall stiffness is functionally compromised in 12-wk-old LPK compared with age-matched normotensive Lewis rats (30), and demonstrate that changes are evident early in the disease process, with SBP elevated by age 6 wk, concurrent with increased isobaric PWV, MCSA, and EM/WS and reduced total elastin density compared with control animals. While PWV increases normally as a function of age (6), the increased isobaric PWV in the 6-wk-old LPK rats corresponded to an E/C ratio comparable to that of 12-wk-old control Lewis animals. These arteriosclerotic changes are consistent with the accelerated aging process associated with renal disease in humans, including increased collagen, degeneration of elastin, increase in wall thickness, and decrease in wall distensibility (21, 26, 35, 46). The very early establishment of arterial stiffness in the LPK model therefore provides a comparable process to that of human CKD, where increased arterial stiffness likely develops before critical creatinine clearances are reached (43).

The administration of perindopril to the LPK rats at 6 wk of age prevented further deterioration in aortic wall structure and function, by preserving the degradation of elastin, retarding collagen synthesis, and ameliorating aortic medial calcification. The impact on calcification is comparable to the work of Armstrong et al. (5), who showed in a rabbit model of diet-induced atherosclerosis that treatment with an ANG II type 1 receptor (AT1R) blocker inhibited arterial calcification. In addition to reducing blood pressure, our results show that long-term treatment with perindopril normalized the mechanical properties of the large artery wall (7). This effect was not observed in the Lewis rats, implicating the RAS in the genesis of large-artery wall stiffness in the LPK. These findings are consistent with work in spontaneously hypertensive rats (SHR), where ACE inhibitors improve large-artery compliance, preventing aortic collagen accumulation independently of blood pressure changes. Of note is that in the SHR, the effects were independent of bradykinin and mediated exclusively through the AT1R, and in the rabbit atherosclerosis model, studies demonstrated a significant upregulation of the AT1R in the calcified regions of the vasculature (5, 8). The effect of perindopril in the LPK may therefore be direct, rather than simply mediated by the reduction of blood pressure.

In CKD, vascular calcification is often accompanied by collagen deposition and a loss of elastin fiber, resulting in gradual stiffening of large arteries (27). While also a feature of
aging, studies in young adults and children with CKD show clinically significant vascular calcification, arguing against this being an age-related process, and recent work has demonstrated that not only does vascular calcification begin early in CKD, but it worsens as the disease progresses (4, 18, 28, 43). Calcification affects large-artery remodeling and is characterized by an enlarged lumen, increase in wall thickness, and a reduction in E/C (14, 31). This occurs because the degraded elastic network in medial elastocalcinosis results in the transfer of wall tension to the stiffer collagen fiber to maintain vascular wall integrity, increasing the stiffness of the aorta (31). However, elastocalcinosis does not induce structural hypertrophy. Thus vascular remodeling and calcification may act in parallel but independently in CKD to cause an increase in arterial stiffness.

The protective effect of perindopril on aortic wall structure and function in the LPK could be due to preventing the downstream actions of ANG II in the vascular wall. Molecules of the ANG II signaling cascade are upregulated within the arterial wall during hypertension and may play a causal role in vascular remodeling (45). Moreover, at the cellular level, ANG II promotes vascular endothelial cell senescence, potentially disturbing the integrity of the vascular wall and promoting vascular injury (39). ANG II also affects the E/C ratio, as inhibition of ANG II production with quinapril prevents the development of aortic hypertrophy and increase in collagen in the SHR through a direct action on smooth muscle cells which is independent of blood pressure reduction (2, 22).

Structurally, WS increases when MAP increases. For EM to provide direct information on the elastic properties of the wall

<table>
<thead>
<tr>
<th>Group</th>
<th>Total elastin density, %</th>
<th>Lamellae elastin number</th>
<th>Lamellae elastin density, %</th>
<th>Interlamellae elastin density, %</th>
<th>Total collagen (5), %</th>
<th>Elastin-to-collagen ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis</td>
<td>6 wk</td>
<td>61 ± 8</td>
<td>8 ± 1</td>
<td>27 ± 9</td>
<td>36 ± 7</td>
<td>24 ± 7</td>
</tr>
<tr>
<td></td>
<td>12 wk</td>
<td>56 ± 11</td>
<td>8 ± 2</td>
<td>26 ± 5</td>
<td>36 ± 9</td>
<td>26 ± 9</td>
</tr>
<tr>
<td></td>
<td>12 wk ACEi</td>
<td>51 ± 10</td>
<td>9 ± 1†</td>
<td>27 ± 4</td>
<td>34 ± 9</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>LPK</td>
<td>6 wk</td>
<td>48 ± 10*</td>
<td>8 ± 2</td>
<td>14 ± 4*</td>
<td>32 ± 9</td>
<td>24 ± 3</td>
</tr>
<tr>
<td></td>
<td>12 wk</td>
<td>49 ± 8</td>
<td>9 ± 1*</td>
<td>21 ± 7</td>
<td>28 ± 13*</td>
<td>32 ± 8</td>
</tr>
<tr>
<td></td>
<td>12 wk ACEi</td>
<td>58 ± 20†</td>
<td>9 ± 1</td>
<td>38 ± 16†</td>
<td>37 ± 16†</td>
<td>25 ± 13†</td>
</tr>
</tbody>
</table>

Values are means ± SD. LPK, Lewis polycystic kidney; ACEi, angiotensin-converting enzyme inhibitor. Morphometric density parameters were calculated based on the relative area occupied (percentage area) by respective structure per unit area of the tissue. Six fields were photographed per each segment of thoracic aorta, and a total of n = 6 animals of each strain were examined. Values tabulated were averages of all 6 slides. Elastin values were obtained automatically using a custom-written Image J cell count macro. For untreated LPK, *P < 0.05 compared with age-matched Lewis control rats. For 12-wk-old treated animals, †P < 0.05 compared with untreated 12-wk-old strain control rats (LPK and Lewis).

Fig. 6. Histological sections stained with Shitaka’s orcein (A and B) and martius/scarlet/blue (C and D) from longitudinal sections of the thoracic descending aorta in untreated LPK (A and C) and perindopril-treated LPK (B and D) 12-wk-old rats. Elastin fiber staining in purple in A and B illustrates the increase in lamellae elastin density and interlamellae elastin density in the perindopril-treated LPK compared with the untreated animals, while martius/scarlet/blue staining in C and D illustrate a reduction in the level of blue collagen content in the perindopril-treated LPK rats. Scale bar = 50 μm.
material and a true representation of wall stiffness independently of vessel geometry, EM was expressed as a function of WS. In the untreated LPK rats, at a given value of WS, EM was much higher, suggesting that at a similar WS imposed by a MAP in the vessel, the aorta of the LPK is significantly stiffer at both 6 and 12 wk of age. EM/WS was reduced in the LPK rats following chronic perindopril treatment and, corresponding to changes in isobaric PWV, EM, and WS, our data also suggest the involvement of elastin and collagen, such that the relative proportion of elastin to collagen was higher in the untreated LPK rats and showed a trend toward amelioration with ACE inhibition. The relative amount of both lamellae and interlamellae elastin was reduced in the LPK rats in relation to the increase in collagen. Following treatment, the relative amount of elastin increased, with no change in the number of elastin lamellae; treatment also modified the increase in collagen density. The influence of perindopril on vascular remodeling may be blood pressure dependent, or it may mediated by non-ANG II mechanisms, as ACE is responsible for both production of ANG II and degradation of the vasoactive bradykinin (10). ACE inhibitors are effective, however, in controlling vascular remodeling, independent of blood pressure, and aortic stiffening as a result of fibrosis can be reversed by ACE inhibition independent of bradykinin preservation (8, 17).

The present study clearly demonstrates the capacity of ACE inhibition to prevent an increase in arterial stiffness associated with CKD and corresponding changes in the structure and composition of the arterial media such as calcification. Our results highlight the need for consideration of the RAS as a key mediator of cardiovascular disease in chronic renal failure, and its contribution to the hypertensive state not only as a direct vasoconstrictor but also as a mediator of vascular remodeling and arteriosclerosis of large arteries. The RAS also impacts other key neurohumoral blood pressure control systems, and future studies are required to delineate the relative contribution of these mechanisms. Of note, however, is our demonstration that perindopril was able to prevent the progression of arterial calcification. This result suggests calcification may be a valid surrogate end point for intervention studies in patients with cystic renal disease and, further, in CKD patients as a population, an issue of recent clinical relevance (26). Indeed the robust response demonstrated in our study indicates a previously unexplored capacity for RAS inhibition to limit aortic calcification and improve cardiovascular function in CKD. The LPK rat is likely to provide a model system where interventions to modify arteriosclerosis can be trialed under conditions that mimic the human condition in a state of natural disease progression.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
K. Ng, C. M. Hildreth, A. P. Avolio, and J. K. Phillips provided the conception and design of research; K. Ng, C. M. Hildreth, and J. K. Phillips performed experiments; K. Ng, C. M. Hildreth, A. P. Avolio, and J. K. Phillips analyzed data; K. Ng, C. M. Hildreth, A. P. Avolio, and J. K. Phillips interpreted results of experiments; K. Ng, C. M. Hildreth, A. P. Avolio, and J. K. Phillips prepared figures; K. Ng, C. M. Hildreth, A. P. Avolio, and J. K. Phillips drafted the manuscript; K. Ng, C. M. Hildreth, A. P. Avolio, and J. K. Phillips edited and revised the manuscript; K. Ng, C. M. Hildreth, A. P. Avolio, and J. K. Phillips approved final version of the manuscript.

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