Effect of medial calcification on vascular function in uremia

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THE INCIDENCE OF CARDIOVASCULAR disease is markedly increased in patients with advanced kidney disease and is associated with changes in vascular function. Arterial compliance is decreased (16) and correlates strongly with death (3). Endothelial function is also abnormal, manifest primarily as decreased endothelium-dependent vasodilatation (9, 10, 12, 20, 21) and may contribute to hypertension and ischemia. Renal failure may affect the function of vascular smooth muscle as well, but the data are disparate with some studies showing enhanced responsiveness and other showing reduced responsiveness to α-adrenergic stimulation (2, 7, 19, 27, 32).

The etiology of vascular dysfunction in renal failure is not known. Histological studies have shown changes in the medial layer of arteries consisting of both hypertrophy and hyperplasia of smooth muscle cells as well as increased matrix (1), which could explain the decrease in compliance. There is also a high incidence of atherosclerosis (8), which might also lower compliance and is associated with endothelial dysfunction. Increased oxidant stress in renal failure (6) is another potential cause of endothelial dysfunction.

Smooth muscle calcification is another common arterial lesion in advanced kidney disease that could also contribute to vascular dysfunction. Although it has been considered to be benign, recent data have shown that vascular calcification correlates with cardiovascular events and death (15). This may be related to a decrease in arterial compliance since increased pulse-wave velocity correlates with vascular calcification and cardiovascular disease in advanced kidney disease (4, 26). However, pulse-wave velocity can be influenced by smooth muscle tone in addition to elasticity (18), and interpretation of these studies is also complicated by the fact that they cannot distinguish between the medial calcification (Monckeberg’s sclerosis) commonly associated with renal failure and the intimal (atherosclerotic) calcification that frequently coexists. Thus medial calcification in uremia has not been directly correlated with arterial compliance, and its effect on endothelial or smooth muscle function is also unclear.

To address the effects of medial vascular calcification in uremia on vascular function, we performed ex vivo measurements of compliance, contraction, and relaxation in calcified and noncalcified aortas from uremic rats and in control aortas from nonuremic rats. Uremia and vascular calcification were induced by feeding a high-phosphate diet containing adenine (24, 30), and comparisons were made between control, pair-fed rats and uremic rats with and without calcification.

METHODS

Animals. Renal failure was induced by feeding adenine as previously described (14), with modifications. Male Sprague-Dawley rats weighing ~300 g were fed a standard rodent diet supplemented with 0.66% phosphorus (from a 2:1 mixture of dibasic and monobasic sodium phosphate). The final content of the diet was 23% protein, 0.95% calcium, and 1.06% phosphorus. To produce renal failure, adenine was added at a concentration of 0.75%. NaCl (0.22%) was added to the drinking water to counteract the salt wasting that occurs with this model. Since rats lose weight on this diet, food was restricted in the control rats so that aggregate weights were similar. For the studies of vessel compliance, the model was altered to produce a wider range of calcification. Dietary protein was reduced to 2.5% (25) or calcitriol (40 ng/kg 3 times/wk) was added to enhance calcification, and dietary phosphorus was lowered to 0.73% to reduce calcification. Rats were killed after 28–30 days. Plasma was obtained by aortic puncture at the bifurcation, and aortas were removed, gently cleaned, and stored in Hanks’ solution. All studies were completed in compliance with protocols reviewed and approved by the Institutional Animal Care and Use Committee.

Vessel studies. One or two rings (4–5 mm in length) were prepared from the straight section of the descending thoracic aortas, without regard to the degree of visible calcification. Contractile and relaxation studies in response to agonists were completed as we have described previously (13). For each aorta, resting tension was adjusted to 50 mN over a 1-h period. All vessels demonstrated maximal active force generation at this tension (data not shown). Data were recorded using PowerLab digital acquisition and analyzed using Chart Software (AD Instruments, Mountain View, CA). Vascular contractility was assessed by generating concentration-response curves to KCl (0–80 mM) and phenylephrine (PE; 0.1 nM–10 μM). Agonist sensitivity was determined by plotting the individual concentration-response
curves as the percentage of maximum and comparing the average of the EC50 values obtained. For comparisons of maximal contraction, isometric force is normalized to cross-sectional area (CSA) which is estimated based on vessel geometry and wet weight [CSA = 2 × wet weight/(circumference)] as described (31). Vascular relaxation was also determined. Following precontraction with 2–5 μM PE, a concentration which yields 80–90% maximum contraction, relaxation responses were examined in response to the endothelium-dependent vasorelaxant acetylcholine (0.1 nM–100 μM) and the endothelium-independent NO-donor sodium nitroprusside (SNP; 0.1 nM–1 μM).

In a separate study, passive mechanical properties of the aorta were assessed using methodologies adapted from Blough et al. (5) as an indication of vessel compliance. Aortic rings were maximally dilated with 30 μM SNP, and tension was applied until a deflection in force measurement was observed. This tension was set as L0, and the length was increased in 25-μm increments while the force was monitored. The length that resulted in maximal force development or resulted in tearing of the vessel was used as an index of the passive mechanical properties of the vessel, or compliance.

After the measurements were completed, the rings were dried and the calcium content was measured as below.

Chemical assays. Plasma urea was assayed enzymatically using urease and glutamate dehydrogenase (ThermoDMA, Arlington, TX). Plasma phosphate was measured colorimetrically by the molybdate method and plasma calcium was determined colorimetrically using 100 μM o-cresolphthalein complexone in 270 mM aminomethylpropanol buffer, pH 10.0, with 5.2 mM 8-hydroxyquinolone added to complex magnesium. Absorbance was measured at 575 nm. To measure aortic calcium content, dried aortas were incubated in 1 M HCl overnight and calcium was measured as described above. Aortas were dried after extraction and weighed, and results are expressed as nanomoles per milligram dry weight. For ex vivo vessel studies, calcium content was measured in the individual rings.

Statistical analysis. Results are presented as means ± SE. EC50 values and maximum force were determined for each sample, and differences between groups were assessed by ANOVA using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Concentration response curves were analyzed using two-way ANOVA followed by Bonferroni tests to compare individual points. Significance was defined as P < 0.05.

RESULTS

Plasma chemistries and aortic calcium content for the animals used in the contraction and relaxation studies are shown in Table 1. These rats were fed standard rat chow (23% protein) supplemented with sodium phosphate to a final content of 1.06% phosphorus with or without adenine to induce renal failure. Plasma urea and phosphate were significantly elevated in adenine-treated rats, with no significant difference between rats with and without aortic calcification. Plasma calcium did not differ between control and uremic rats or between uremic rats with and without aortic calcification.

Aortic calcium content was variable in uremic rats fed standard rat chow with 1.06% phosphorus, with only about one-half the aortas exhibiting any calcification. Aortas were considered to be calcified when the calcium content was >37 nmol/mg, which is three SD above the mean content in whole aortas from nonuremic rats. There was a clear separation between calcified and noncalcified aortas, with the range in calcified aortas being 577–4,794 nmol/mg. Calcification was visually apparent when the calcium content exceeded 300 nmol/mg and was diffuse and severe when the content exceeded 1,500 nmol/mg. Staining of the aortas for calcification (von Kossa stain) is shown in Fig. 1. There was no staining in control or noncalcified uremic aortas, and they appeared oth-

Table 1. Biochemical values in control and uremic rats fed a 23% protein and 1.06% phosphorus diet

<table>
<thead>
<tr>
<th></th>
<th>Control Rats (n = 6)</th>
<th>Noncalcified (n = 5)</th>
<th>Calcified (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic calcium, nmol/mg</td>
<td>20.8 ± 2.2</td>
<td>24.9 ± 2.7</td>
<td>2453 ± 746*</td>
</tr>
<tr>
<td>Plasma BUN, mg/dl</td>
<td>14.4 ± 3.3</td>
<td>58.7 ± 10.6†</td>
<td>65.4 ± 8.6†</td>
</tr>
<tr>
<td>Plasma phosphorus, mM</td>
<td>0.82 ± 0.21</td>
<td>2.64 ± 0.75†</td>
<td>2.57 ± 0.63†</td>
</tr>
<tr>
<td>Plasma calcium, mM</td>
<td>1.87 ± 0.56</td>
<td>1.80 ± 0.25</td>
<td>1.78 ± 0.17</td>
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Values are means ± SE. BUN, blood urea nitrogen. *P < 0.002 vs. noncalcified or control. †P < 0.0001 vs. control.

Fig. 1. Aortic histology in control and uremic rats fed a 23% protein and 1.06% phosphorus diet. Aortas were stained by the von Kossa method, in which calcium phosphate appears black. No calcification was observed in aortas from control, nonuremic rats (A), and in uremic aortas with a normal Ca content (B). Aortas with increased calcium content exhibited both patchy (C) and diffuse, circumferential (D) calcification restricted to the medial layer. The same magnification was used for all images.
eriwise similar. Moderately calcified aortas showed patches of medial calcification, with the remainder of the aorta appearing normal. Heavily calcified aortas showed calcification within the vascular media that was histologically similar but fully circumferential. The fracturing that is present is an artifact of the tissue preparation.

The heterogeneous distribution of the calcification makes it impossible to assign a calcium content to each image, but these sections are representative of the rings used for measurement of contraction and relaxation. Although the vascular wall appears thickened in portions of Fig. 1D, heavy calcification was usually associated with thinning of the wall and dilatation, which is readily apparent in the calcified portion of Fig. 1C. Medial thickness was $130 \pm 14$ and $134 \pm 5 \mu m$ in control and noncalcified uremic aortas, respectively, indicating that smooth muscle mass was not altered in uremic aortas, but sensitivity was significantly reduced. There were no significant differences between calcified and noncalcified aortas.

Smooth muscle contractile function was investigated by measuring force generation in response to depolarization (using KCl and to the $\alpha$-adrenoceptor agonist PE. Sensitivity to KCl was increased in uremic aortas (Fig. 2), with no difference between noncalcified and calcified aortas ($EC_{50} 28.1 \pm 1.0$, $18.0 \pm 1.7$, $17.5 \pm 2.8$ nM in control, uremic noncalcified, and uremic calcified, respectively) ($P < 0.01$ vs. control for either noncalcified or calcified uremic aortas). In addition, the maximum force per cross-sectional area obtained with KCl was markedly decreased in uremic aortas, again with no difference between noncalcified and calcified aortas ($2.38 \pm 0.33$, $0.87 \pm 0.14$, and $1.01 \pm 0.43$ mN/mm$^2$ in control, uremic noncalcified, and uremic calcified, respectively; $P < 0.05$ vs. control; $n = 6 \sim 8$). In the case of PE, there were no changes in sensitivity (Fig. 3) but the same profound reduction in maximum force was observed ($2.60 \pm 0.56$, $1.12 \pm 0.17$, and $1.23 \pm 0.51$ mN/mm$^2$ in control, uremic noncalcified, and uremic calcified, respectively) ($P < 0.05$ vs. control for either noncalcified or calcified uremic aortas; $n = 6 \sim 8$). Again, there were no differences between calcified and noncalcified aortas in response to PE.

Vasorelaxation studies are shown in Fig. 4. Relaxation responses to acetylcholine, an endothelium-dependent vasodilator, were profoundly depressed in uremic aortas. Maximal relaxation with SNP, a direct smooth muscle dilator, was not altered in uremic aortas, but sensitivity was significantly reduced. There were no significant differences between calcified and noncalcified aortas.

Fig. 2. Contractile response of uremic aortas to KCl. Top: absolute force as a function of KCl concentration. Bottom: relative force as a function of KCl concentration. ○, Control ($n = 6$); □, uremic, noncalcified ($n = 8$); ■, uremic, calcified ($n = 6$). Rats were fed a 23% protein and 1.06% phosphorus diet. CSA, cross-sectional area. *$P < 0.05$ compared with control by 2-way, repeated-measures ANOVA followed by Bonferroni correction.

Fig. 3. Contractile response of uremic aortas to phenylephrine (PE). Top: absolute force as a function of PE concentration. Bottom: relative force as a function of PE concentration. ○, Control ($n = 6$); □, uremic, noncalcified ($n = 8$); ■, uremic, calcified ($n = 6$). Rats were fed a 23% protein and 1.06% phosphorus diet. *$P < 0.05$ compared with control by 2-way, repeated-measures ANOVA followed by Bonferroni correction.
Aortic compliance was measured directly ex vivo as tension vs. distance in aortic rings maximally relaxed with SNP. Compliance was reduced >50%, but the aortas were either severely calcified or not calcified at all. To determine whether lesser degrees of calcification also reduce compliance, changes were made in the model to obtain intermediate degrees of calcification. First, dietary protein was reduced to 2.5%, which increases aortic calcification in adenine-treated rats by an unknown mechanism (25). This increased the prevalence of media calcification and yielded some aortas with intermediate calcium contents. Greater success was achieved by administering graded doses of calcitriol (up to 40 ng/kg 3 times/wk) to rats fed standard chow with 0.73% phosphorus. There was no apparent difference in compliance between aortic rings with similar calcium contents generated with the different models, so the data were combined to show compliance as a function of calcium content. As shown in Fig. 5, compliance was reduced in almost all calcified vessels and was inversely related to the degree of calcification. The regression line for the data is 358 ± 0.024 × aortic calcium content (r = 0.86), and the slope is significantly less than zero by linear regression (P < 0.0001). Insert: values for aortas with calcium contents <900 nmol/mg. Nonuremic aortas. Data are from rats fed a 23 or 2.5% protein diet with 1.06% phosphorus diet and from rats fed a 23% protein, 0.73% phosphorus diet, and treated with calcitriol up to 40 ng/kg 3 times/wk.
calcification to aortic stiffness has been uncertain. This study indicates that at least some of the reduced arterial compliance in chronic kidney disease and end-stage renal disease can be explained by medial calcification. This is consistent with the correlation between pulse-wave velocity and medial calcification in nonuremic rats treated with nicotine and toxic doses of vitamin D (22) and with the increased pulse wave velocity in uremic rats treated with doses of doxercalciferol that produce aortic calcification (23).

A recent study in uremic ApoE−/− and wild-type mice, in which most of the aortic calcification was intimal, found no correlation between pulse-wave velocity and aortic calcification (17). This finding and the decreased compliance observed in the current study suggest that only medial calcification reduces arterial compliance, which may be related to the loss of elastin in medial calcification (22). Pulse-wave velocity was increased in uremic mice in the absence of calcification or other structural changes in the aorta, demonstrating that pulse-wave velocity is not necessarily indicative of changes in arterial structure. In the current study, compliance was not altered in uremic aortas that were not calcified, possibly due to different hemodynamics, different species, or the shorter duration of uremia.

Although marked changes in smooth muscle and endothelial function were observed in calcified vessels, the changes were qualitatively and quantitatively the same in noncalcified uremic vessels, indicating that the effects on vascular function were the result of uremia rather than calcification. It is possible that intimal calcification, which did not occur in this study, can alter vascular function. A severe reduction in maximal force generation in calcified aortas was also noted in a previous study of rats made uremic by 5/6 nephrectomy and given a very high calcium diet and high doses of calcitriol (29). The calcification was exclusively medial and histologically identical to that obtained in adenine-fed rats in this study. Both calcification and force generation were improved in rats treated with etidronate, suggesting that medial calcification affects smooth muscle function. However, noncalcified uremic aortas were not studied and calcium content was not measured or correlated with force generation. In another study, treatment of uremic rats with paricalcitol increased aortic calcification and reduced force generation in superior mesenteric arteries (11), but it is not clear whether these vessels were calcified. Vasodilatation of these arteries was not altered by paricalcitol, consistent with the present results showing no effect of medial calcification on relaxation.

The lack of effect of medial calcification on vascular function despite extensive and grossly visible calcification with reduced compliance may be explained by the fact that contraction and relaxation were measured under isometric conditions in which very little wall motion is necessary to measure force. It is also possible that the profound effects of uremia alone on contraction and relaxation masked any additional effect of calcification. Changes in vessel function might therefore be more apparent when medial calcification occurs in the absence of renal failure, such as in diabetes and aging. Hemodynamics are also determined by chronic changes in vessel caliber related to remodeling of smooth muscle, and this could be altered by medial calcification in the absence of changes in acute vaso-motor responses.

This study demonstrated marked alterations in both smooth muscle and endothelial function in uremic rats. Maximal force was substantially reduced in response to either KCl or PE, and sensitivity to KCl was increased. Previous studies of arterial function in animals and in humans with renal failure have yielded contradictory results, with both increased (2, 32) and decreased (7, 11, 19, 27) α-adrenergic sensitivity noted. The mechanisms underlying the changes in smooth muscle function in uremia have not been investigated. The severe reduction in maximum force in the absence of reduced smooth muscle mass indicates a significant impairment in contractile capacity. The underlying mechanism is unknown but could involve changes in vasoactive substances or the intracellular signaling cascades that regulate calcium sensitivity in vascular smooth muscle (28).

Relaxation in response to acetylcholine, an endothelium-dependent dilator, was severely impaired in the uremic rats in this study. Although relaxation to sodium nitroprusside, a direct vasodilator, was also impaired, the reduction was not severe and could not entirely explain the impaired response to acetylcholine. This impairment in endothelium-dependent relaxation has been observed in prior studies, both in animals (10, 11) and in humans (21) ex vivo, and in humans in vivo (9, 12, 20), but the impaired direct vasodilation has not previously been described.

Heavily calcified aortas exhibited significant dilatation, likely due to the destruction of elastin that has been demonstrated in the vitamin D-nicotine model of medial calcification (22). However, this is unlikely to have artifactually lowered compliance. Measurements of compliance were based on an initial length that was set for each ring based on the development of tension and the fact that distance was not normalized to initial distance would tend to increase apparent compliance in dilated aortas. Measurements of force generation were also based on an initial tension and normalized to cross-sectional surface area, which would tend to lower force generation in dilated aortas. Measurements of vasorelaxation are independent of vessel geometry.

Caution must be exercised in extrapolating these results to patients with kidney disease. Severe uremia and vascular calcification developed within a month in this model, clearly faster than the development of vascular calcification in humans and without time for remodeling of the vessel wall that could affect vascular function in chronic kidney disease. However, this model does demonstrate that medial vascular calcification can significantly reduce arterial compliance without affecting the function of smooth muscle or endothelium.
VASCULAR CALCIFICATION AND FUNCTION IN UREMIA


